COLD TOLERANCE IN *ARABIDOPSIS KAMCHATICA* AND RELATED SPECIES

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COLD TOLERANCE IN *ARABIDOPSIS KAMCHATICA* AND RELATED SPECIES

A

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ABSTRACT

Cold is a major limiting factor in the development and distribution of plants. Many plants increase cold tolerance via cold acclimation. We determined the cold tolerance strategy of *Arabidopsis thaliana* and *A. kamchatica* by comparing the extent that plants cooled below the freezing point without freezing, the supercooling capacity, to the temperatures at which 50% of freeze damage occurred (LT50). In *A. kamchatica* LT50 and supercooling values were similar before cold acclimation; after acclimation LT50 was much colder than supercooling, indicating non-acclimated *A. kamchatica* avoids freezing by supercooling but after acclimation appears to tolerate freezing. In *A. thaliana*, LT50 and supercooling were not different, regardless of acclimation, indicating this species avoids freezing year-round. We compared cold hardiness in populations from five *Arabidopsis* taxa by measuring freeze induced electrolyte leakage. There were differences among taxa; *A. kamchatica*, *A. lyrata* subspecies *lyrata*, and *A. lyrata* subspecies *petraea* were more cold tolerant, whereas *A. thaliana* and *A. halleri* subspecies *gemmifera* were less tolerant. There was no correlation between latitude of population origin and cold tolerance for any of the species we tested. Our results indicate a shared evolutionary history may be more important than latitude of origin as a predictor of cold hardiness.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

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GENERAL INTRODUCTION

Temperature is considered to be a major limiting factor for plant growth and geographic distribution (WEIGEND and LUEBERT, 2009). Tolerance to cold varies widely across the plant kingdom, with some species becoming severely damaged at low, non-freezing temperatures, while others survive the extreme cold in places such as the Arctic and Antarctic. Cold tolerance also varies seasonally (WEIGEND and LUEBERT, 2009), so if a plant is not ready for it, low temperatures can damage tissues even in species considered to be cold hardy. Many plant species use a complex process called cold acclimation to increase their ability to survive cold temperatures after exposure to low, but non-freezing temperatures (THOMASHOW, 1999). Cold acclimation involves many biochemical and physiological changes, such as the increase of antioxidants and osmolytes, cessation of growth, and changes in membrane composition (XIN and BROWSE, 2000). Some species have a high capacity to cold acclimate while others only acclimate a few degrees or not at all (WEIGEND and LUEBERT, 2009).

When plants are exposed to subzero temperatures, the liquid within the plant can freeze, starting with extracellular water because these fluids have a warmer freezing point than fluids in intracellular spaces (BERTRAND and CASTONGUAY, 2003). As extracellular fluids are freezing, the intracellular water is attracted to the lower water potential of the extracellular ice, thus dehydrating the cell and causing membrane damage (THOMASHOW, 1999). Intracellular freezing is typically lethal (ATICI and NALBANTOGLU, 2003).

There are two main strategies for plant cold tolerance: some species avoid freezing, while others can tolerate extracellular freezing (WEIGEND and LUEBERT, 2009). Many plants that avoid freezing depress their freezing point via supercooling (ATICI and NALBANTOGLU, 2003) and would be damaged or killed if temperatures dropped below their capacity to supercool. While many herbaceous plants supercool, this is likely only a significant freeze avoidance mechanism in milder climates where a few degrees of protection are sufficient (WEIGEND and LUEBERT, 2009). In contrast, freeze tolerant plants allow extracellular fluids to freeze while avoiding intracellular ice formation and

protect against intra- and intercellular ice formation by producing solutes and amino acids (ATICI and NALBANTOGLU, 2003). Some freeze tolerant plants have the ability to produce antifreeze proteins after cold acclimation (DUMAN and OLSEN, 1993). Antifreeze proteins are thought to help prevent recrystallization of small ice crystals into larger, more damaging crystals (DUMAN and OLSEN, 1993) and inhibit ice growth (GRIFFITH *et al.*, 2005).

The process of cold acclimation has been the subject of intense study and much has been revealed about the genetic and physiological processes that occur when plants are exposed to freezing temperatures (THOMASHOW, 2001). The use of *Arabidopsis thaliana* as a model species for cold tolerance has greatly increased our understanding of the molecular and physiological changes that occur upon exposure to cold (FRANKLIN and WHITELAM, 2007; GILMOUR *et al.*, 1988; THOMASHOW, 2010). Other members of the *Arabidopsis* genus have also been used as a model for the study of evolution (HOFFMANN, 2005), molecular and population genetics (ANSELL *et al.*, 2010), as well as plant physiology (CLAUSS and KOCH, 2006), and adaptation to environmental stress (DAVEY *et al.*, 2009). There is a wealth of evolutionary and ecological tools available for *Arabidopsis* species (BECK *et al.*, 2007). Members of the *Arabidopsis* genus provide a unique opportunity to compare traits in closely related species where the evolutionary relationships and range distributions are known (BECK *et al.*, 2007). While the molecular and physiological aspects of plant cold hardiness have been extensively investigated across the plant kingdom, many of the similarities and differences between related species remain unknown because few studies have tested multiple species from within the same genus to determine how evolutionarily conserved cold tolerance limits are among related species. The range distributions for *Arabidopsis* species are quite variable in size and often overlap (HOFFMANN, 2005), making the comparison of cold tolerance among species from this genus particularly interesting.

Adaptations for tolerance to the cold play an important role in determining range limits of many species (PITHER, 2003). At high latitudes, plants often experience a

broader range of temperatures than those of lower latitudes, meaning fewer species can survive in northern climates (CRIDDLE *et al.*, 1994) where global climate change is predicted to have its most extreme effects (BOKHORST *et al.*, 2010). For plants to expand their range and tolerate a changing climate, they must be able to adapt to new conditions. Many plants are sensitive to temperature or nutrient changes which may lead to shifts in species distributions. As global temperatures warm, the capacity of arctic and subarctic flora to successfully adapt will determine their ability to withstand not only new temperature regimes, but also invasion from other species. It has been predicted that warming temperatures may allow for a general northward migration of many species, however species from warmer climates may be less capable of tolerating unpredictable temperature extremes found in northern climates (JALILI *et al.*, 2010).

Members of the *Arabidopsis* genus usually have a well-defined basal rosette (AL-SHEHBAZ and O'KANE, 2002). Many populations of *A. thaliana* exhibit a winter annual life cycle, meaning that the plants in these populations overwinter as rosettes and flower in spring (GRIFFITH *et al.*, 2004; NORDBORG and BERGELSON, 1999). Most other *Arabidopsis* species have a biennial or perennial life cycle, meaning plants also overwinter as rosettes (AL-SHEHBAZ and O'KANE, 2002). *Arabidopsis* species are primarily found in the cold and temperate climate zones, often in cold and dry areas (HOFFMANN, 2005). Because herbaceous plants from northern regions often depend on snow cover to insulate overwintering structures against severe cold (BERTRAND and CASTONGUAY, 2003), short stature and sufficient snow cover are likely to be important contributing factors for the over-winter survival of *Arabidopsis* plants from northern populations.

Understanding the nature of freezing tolerance and cold acclimation is an active area of research partially because temperature is one of the limiting factors in potential locations for growing crops (THOMASHOW, 1999). Future climate changes are predicted to include effects beyond changing global temperatures; some models predict there will be an increase in freezing rain, melting and thawing cycles, and less snow cover; all of

which would have a strong influence on the over-winter survival of plants from cold climates (BERTRAND and CASTONGUAY, 2003). While it may seem that warming temperatures would be beneficial for the agriculture industry, the predicted increase in the frequency of extreme weather events and changing temperature regimes may instead increase economic losses. Cold-induced damage is a major source of crop loss (BURKE *et al.,* 1976) and new types of stress such as increased exposure to cold because of unpredictable snow melt or ice encapsulation may lead to increased losses as many herbaceous plants depend on snow cover for insulation from cold temperatures (BERTRAND and CASTONGUAY, 2003). Freeze resistance is lowest during active periods of growth (LARCHER *et al.,* 2010), therefore an increase in freeze resistance during the growing season may lead to a reduction in crop losses due to unseasonable frosts.

This master's project investigated cold tolerance in *Arabidopsis* species. The cold tolerance strategies of *A. thaliana* and *A. kamchatica* were determined by quantifying and comparing the freeze damage and supercooling capacity for each species. This study builds on what is already known about cold tolerance in *A. thaliana,* in an attempt to determine if the same cold tolerance strategy is found in a closely related species. We then quantified freeze damage for five *Arabidopsis* taxa and compared several populations from across the distribution of four of the species to ascertain if there was a relationship between latitude of origin and cold tolerance. This study contributes to our understanding of the resilience of cold adapted organisms to withstand the rapid climate warming currently occurring in the far North. Studying the evolution of cold tolerance across a wide latitudinal range will help us to understand both historic and future species range expansions and contractions brought about by changes in climate.

Objective of this thesis

The main objective was to investigate cold hardiness in *A. kamchatica* in relation to other members of the *Arabidopsis* genus. Specifically, this study addressed the following objectives:

In Chapter 1: I compare LT50 and supercooling in *A. kamchatica* and *A. thaliana* to predict the over-winter survival strategy of each species. These species were selected to enable us to compare cold tolerance in a well-studied model species, *A. thaliana*, with that of a close relative that has a more northern distribution, *A. kamchatica,* to see how similar the strategies of these closely related species are.

In Chapter 2: I compare cold induced electrolyte leakage rates in populations from several members of the *Arabidopsis* genus to look for differences among species and within populations from different latitudes to determine if there was a correlation between latitude of population origin and cold tolerance.

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Chapter 1: Cold tolerance strategy of *Arabidopsis kamchatica*[1]

ABSTRACT

Premise of Study: Changes in climate at northern latitudes have been predicted to include more erratic weather events during the growing season, which may cause widespread changes to plant distributions. Understanding more about freeze resistance both during the growing season and over winter is essential for predicting how species will respond to changes in climate.

Methods: This study examined the temperature at which freeze damage occurs (LT50) and supercooling in *Arabidopsis kamchatica* and its close relative, *A. thaliana.* We studied both non-acclimated and acclimated plants to understand more about the freeze resistance strategy of these species. To further investigate differences across the latitudinal range of *A. kamchatica*, we compared supercooling and LT50 in plants from a northern, mid-latitude and southern population of the species range.

Key Results: Before and after acclimation, LT50 and supercooling were lower for *A. kamchatica* than *A. thaliana*. In *A. kamchatica,* the LT50 and supercooling values are similar before acclimation, however the LT50 is much colder than the supercooling capacity after acclimation. In *A. thaliana* the LT50 values are similar to the supercooling capacity both before and after acclimation. The mid-latitude population of *A. kamchatica* had the lowest LT50 before and after acclimation.

Conclusions: Before cold acclimation, *A. kamchatica* has the potential to avoid freezing by supercooling but likely has a freeze tolerant strategy after cold acclimation. In contrast, *A. thaliana* appears to avoid freezing year round. The LT50 of populations of *A. kamchatica* were different; however the differences may be correlated with climate variability rather than a latitudinal cline. With its deeper cold tolerance and higher acclimation capacity, *A. kamchatica* cold tolerance research provides a useful complement to research in *A. thaliana*.

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[1] Armstrong, J., D. E. Wolf, N. Takebayashi. Cold tolerance in *Arabidopsis kamchatica*. Prepared for submission to American Journal of Botany.

INTRODUCTION

Cold temperatures affect plant productivity and growth, and for many species, temperature is a habitat limiting parameter (HOFFMANN, 2002). For some plants a slight frost is lethal, while others survive temperatures colder than -40° C. Many plants respond to exposure to low, non-freezing temperatures with a process called cold acclimation that leads to an increase in cold tolerance. This increase in cold tolerance is the result of a cascade of biochemical changes including: increased solute concentrations, changes in membrane lipid composition, and the up- or down-regulation of hundreds of proteins (GILMOUR *et al.,* 1988; THOMASHOW, 1999). While many plants have the ability to cold acclimate, the degree of acclimation varies among species (BURKE *et al.,* 1976) and with the maturity stage of the plant (BOORSE *et al.,* 1998).

Measuring plant freeze damage before cold acclimation gives an estimate of the plant's ability to survive a rapid freezing event during the growing season; comparing those measurements to those taken after acclimation gives an estimate of the plant's ability to increase its freeze tolerance during winter (HANNAH *et al.,* 2006). For many plants, surviving frost during the growing season is likely to be as critical as surviving more severe cold temperatures during winter (SIERRA-ALMEIDA *et al.,* 2009). Because cold is a widespread environmental stress, it is important to understand freezing and freeze damage in plants. This study combines several approaches to examine cold tolerance in *Arabidopsis kamchatica,* a species with a northern distribution, and compares this species with a close relative, *A. thaliana,* a species with a more southern distribution.

Over-winter survival for plants in sub-zero temperatures requires plants to have some mechanisms to either avoid or tolerate ice formation within tissues. Freeze avoidant plants are able to cool extensively below the freezing point without freezing, a process known as supercooling. In freeze avoiding plants, the temperature at which ice forms (the ice nucleation temperature), and the temperature at which 50% freeze damage to tissues occurs (the LT50), are similar (HEKNEBY *et al.,* 2006). While almost all plants supercool to some extent, most herbaceous plants do not supercool to extremely low temperatures (BURKE *et al.,* 1976) and it is thought that for many species supercooling is not a stable

long-term over-winter strategy and is more likely to be useful for short-term freeze avoidance (SAKAI and LARCHER, 1987), for instance surviving a cold snap during the growing season. This is because the extent of supercooling influences ice nucleation and proliferation, and high amounts of supercooling can lead to rapid freezing (BURKE *et al.*, 1976). Supercooling is however common in high altitude and tropical environments, and areas with a seasonal climate (BRAVO *et al.*, 2001; REYES-DIAZ *et al.*, 2006). Persistent supercooling has been reported in some species (NEUNER and BANNISTER, 1995) and may be most effective in environments with relatively stable temperature regimes (LIPP *et al.*, 1994). Therefore, for some species supercooling is a very important component for both surviving frosts during the growing season and for over-winter survival.

Supercooling capacity and the ice formation temperature in the leaf can be measured in the laboratory by several methods including visualization with infrared video thermography (PEARCE, 2001) and thermal analysis (REYES-DIAZ *et al.*, 2006). For thermal analysis, a thermocouple is placed at the leaf surface to measure the temperature; freezing causes an exothermic event because heat is released as the liquid water turns to ice. The temperature at which this exothermic event begins is known as the supercooling point or ice nucleation temperature (HEKNEBY *et al.*, 2006).

On the other hand, some species of plants tolerate freezing. While intracellular ice formation is generally lethal (FUJIKAWA *et al.*, 1999; GUY, 2003), some plants can tolerate the formation of ice in extracellular spaces without tissue damage (LIPP *et al.*, 1994). Freeze tolerant plants often freeze at high sub-zero temperatures in order to better control ice proliferation (SAKAI and LARCHER, 1987) and the supercooling point is often far warmer than the LT50 (LIPP *et al.*, 1994). Freeze damage can be quantified by exposing excised leaves or whole plants to freezing temperatures and measuring the resulting damage. With excised leaves, the amount of electrolyte leakage after cold exposure is measured to determine the amount of freeze damage and the loss of 50% of electrolytes is considered to be the LT50 or lethal temperature (GRIFFITH *et al.*, 2004). This method has the benefit over whole plant survival assays in that it is possible to test

the same individual multiple times, particularly before and after cold acclimation, and at multiple temperatures.

Some freeze tolerant plants are capable of producing antifreeze proteins (HUANG *et al.*, 2002). Antifreeze proteins were first identified in teleost fish (GRIFFITH and EWART, 1995) in 1957 and have since been found in insects and many other terrestrial arthropods (HUANG *et al.*, 2002). Contrary to the strategy found in plants, most antifreeze producing fish and insects avoid freezing (HUANG *et al.*, 2002), and antifreeze proteins have been shown to promote supercooling; depress the freezing point by up to 1.5 to 6° C in fish and insects, respectively; and inhibit the recrystallization of ice crystals (GRIFFITH *et al.*, 2005; HUANG *et al.*, 2002). Antifreeze activity was discovered in plants in 1992 (URRUTIA *et al.*, 1992), and has since been identified in more than 27 species (GRIFFITH and EWART, 1995; HUANG *et al.*, 2002; THOMASHOW, 1998) and in many different parts of overwintering plants including seeds, petioles, flowers, bark, and branches, amongst others (GRIFFITH and YAISH, 2004). Plant antifreeze proteins are only expressed after cold acclimation and appear to have a limited capacity to depress the freezing point, generally 0.2 to 0.5° C (HUANG *et al.*, 2002; URRUTIA *et al.*, 1992). In plants, it appears that the role of these proteins is not to prevent freezing because they have a minimal effect on the freezing point (PEARCE, 2001; URRUTIA *et al.*, 1992); instead antifreeze proteins are incorporated into the ice crystal lattice (GRIFFITH and YAISH, 2004) and act to control freezing (GRIFFITH *et al.*, 2005), prevent recrystallization (GRIFFITH *et al.*, 2004), and may change the shape of the ice crystal (URRUTIA *et al.*, 1992), thereby protecting the plant from freezing injury.

Cold tolerance has been well studied in the model organism *A. thaliana*. Studies of *A. thaliana* from around the world indicate that there is variation among populations in cold tolerance measured in the lab, which is correlated with both latitude and the average minimum temperature experienced in their natural habitat (HANNAH *et al.*, 2006; ZHEN and UNGERER, 2008; ZUTHER *et al.*, 2012). There have been conflicting opinions about the over-winter strategy in *A. thaliana*. Two studies that investigated only LT50 concluded that it is freeze tolerant (GILMOUR *et al.*, 1988; HANNAH *et al.*, 2006), but

another study concluded that it avoids freezing by supercooling (REYES-DIAZ *et al.*, 2006). This disparity is mainly due to a difference in the definition of freeze avoidance vs. tolerance. Both studies found similar LT50 values (-7 to -10° C, depending on accession) but the Reyes-Diaz study also used thermal analysis to measure the temperature of ice nucleation (supercooling point) and compared this to the LT50 temperature. Testing leaf freeze damage alone is not enough to determine whether a plant has a freeze tolerant or freeze avoiding strategy, it is necessary to also determine the supercooling point (REYES-DIAZ *et al.*, 2006).

To determine whether a plant has a strategy of freeze avoidance or freeze tolerance, it is necessary to compare the temperature where leaf damage occurs to the temperature of ice nucleation. Freeze tolerance signifies that an organism not only has the ability to tolerate some ice formation but that it can also survive after exposure to the freezing temperatures (REYES-DIAZ *et al.*, 2006). Testing just freeze damage would be informative about the amount of damage a plant would exhibit at a given temperature, but not what temperature would actually cause the plant to freeze. Conversely, only measuring supercooling would be informative about the temperature at which the plant would freeze but would not be informative about survival after exposure to cold. By measuring both supercooling capacity and LT50, it is possible to examine both freeze damage and survival at the temperatures at which ice formation occurs (REYES-DIAZ *et al.*, 2006). Studies have found that plants with a supercooling capacity at or near the LT50 cannot tolerate freezing (HEKNEBY *et al.*, 2006; SIERRA-ALMEIDA *et al.*, 2009), while plants with a LT50 lower than supercooling are freezing tolerant (SIERRA-ALMEIDA *et al.*, 2009).

Although cold tolerance in *A. thaliana* has been well studied, its natural range doesn't include extremely cold environments (Fig 1.1). The wide latitudinal range and northern distribution of a close relative, *A. kamchatica,* makes this species ideal for the study of extreme cold tolerance and natural variation at high latitudes. *A. kamchatica* and winter-annual *A. thaliana* both overwinter as rosettes, and in northern regions the plants would often be insulated by a layer of snow. This snow is likely an important part of the

over-winter survival for northern plants because temperatures under the snow rarely drop below -10° C, even when air temperatures fall below -40° C (Fig 1.2).

To investigate the cold tolerance strategy in *A. kamchatica* and *A. thaliana*, it was necessary to determine the amount of freeze induced electrolyte leakage and the supercooling capacity of these species before and after cold acclimation. For these assays, excised leaves were cooled to a range of temperatures that are encountered under the snow in Fairbanks, AK and the LT50 of plants was estimated. Plants were then exposed to more extreme temperature ranges in order to measure freeze damage at temperatures colder than the estimated LT50. A common garden near the northern edge of the species range was used to determine whether there are differences in over-winter survival and fitness of *A. kamchatica* plants that originated from different latitudes. Fluids from *A. kamchatica* leaves were tested before and after cold acclimation to look for thermal hysteresis activity, which would indicate a potential for antifreeze proteins in this species.

The goal of this study was to determine 1) whether there are differences between *A. thaliana* and *A. kamchatica* in supercooling and leaf freezing damage due to cold temperatures, 2) if *A. kamchatica* plants cold acclimate after exposure to low, non-freezing temperatures, 3) does *A. kamchatica* use a strategy of freeze avoidance or freeze tolerance?

MATERIALS AND METHODS

Study system—

Arabidopsis thaliana has been widely used as a model species for understanding cold tolerance in plants (HANNAH *et al.*, 2006; THOMASHOW, 1999; XIN and BROWSE, 2000). *A. thaliana* is a diploid, highly self-fertilizing species with a wide climatic and geographic distribution throughout the United States, Europe, and Asia (HOFFMANN, 2005). Plants throughout the range may be either summer annuals or winter annuals (GRIFFITH *et al.*, 2004). Winter annuals germinate in the fall and overwinter as rosettes

under the snow and flower in spring (GRIFFITH *et al.,* 2004). *A. kamchatica* (Fisch. Ex DC.) K. Shimizu & Kudoh is an allotetraploid, self-compatible, perennial, that also overwinters as a rosette and flowers shortly after snowmelt. Although leaves are typically red and leathery after snowmelt, the leaves survive winter and may contribute resources needed for spring growth and flowering as there is no bulb or corm for resource storage. This species likely originated from the natural hybridization of *Arabidopsis halleri* and *Arabidopsis lyrata* (SHIMIZU-INATSUGI *et al.,* 2009). *A. kamchatica* has a smaller but more northern distribution than *A. thaliana*, and is found in eastern Russia, Asia, Alaska and Canada (HOFFMANN, 2005). Both of these herbaceous species are found in disturbed areas, such as roadsides, gravel pits, rocky slopes and along creeks (AL-SHEHBAZ and O'KANE, 2002). *A. thaliana* can be found at elevations up to 4,250 m, while *A. kamchatica* plants are only found up to 3,500 m (AL-SHEHBAZ and O'KANE, 2002).

Plant material—

Arabidopsis kamchatica—

During fall 2007, seeds were collected in the field from 60 plants from each of the three populations spanning the entire latitudinal range of the species in North America. The southern population was located at Elk River in Strathcona Park, Vancouver Island, British Columbia, Canada (49°49'74 N 125°52'36 W); the mid-latitude population was at Portage Glacier, Alaska, USA (60°47'25 N 148°54'06 W); and the northern population was at Ptarmigan Creek, Alaska, USA (65°27'12 N 145°30'27 W). Since this species is fully self-fertile and autogamous in the greenhouse, maternal families may be full-sib self-fertilized families. However, since self-fertilization rates have not yet been measured in the field, we do not know what fraction of offspring may be the result of outcrossing.

Arabidopsis thaliana—

Seeds from 5 accessions (Col, Ler, Cvi, Seattle, British Columbia) of *A. thaliana* were obtained from the Arabidopsis Biological Resource Center at Ohio State University. Each accession represents a self-fertilized lineage, and individuals within an accession are expected to be genetically homogeneous.

Seeds were planted in Scott's Miracle-Gro Seed Starting Potting Mix and were transplanted into a 1:1:1 vermiculite:perlite:coconut core mix after germination. *A. kamchatica* seeds were cold stratified at 4° C for 3 weeks to promote germination, though a second stratification was necessary for some Strathcona Park seeds to germinate. *A. thaliana* seeds were stratified at 4° C for 2 weeks. *A. kamchatica* was grown in 21 cm conical tubes (Cone-tainers, Stuewe and Sons, Tangent, OR, USA) for all laboratory experiments, while *A. thaliana* was grown in 15 cm pots.

Electrolyte leakage assay—

Plants were watered 24 hours before the electrolyte leakage assay to reduce variation based on leaf hydration state. At the same time each morning, rosette leaves of similar age and size were removed from each plant, with one leaf for each temperature to be tested. Leaves were placed in the bottom of a 10x13 mm glass tube, which was immersed in a NesLab programmable circulating ethanol bath (Portsmouth, New Hampshire, USA). The tubes were equilibrated at 0° C for 30 minutes and cooled at a rate of 1° C h^{-1} to -11° C. Cooling was paused at -2° C and small ice chips were added to each tube to nucleate the samples. Tubes were removed every degree from -6 to -11° C. The tubes were allowed to thaw at 4° C for at least 16 hours, upon which 5.4 mL of deionized water was added to each tube and the tubes were shaken in a water bath at room temperature for 1 hour. The initial electrolyte leakage (EL_I) was measured using an Oakton CON6 conductivity meter (Vernon Hills, Illinois, USA). Because of concerns that the liquid in tubes could boil over in the autoclave, half of the leachate was poured into a 2nd tube and the original tubes were autoclaved. The leachate was returned to the original tubes from the 2nd tubes and the contents were shaken again at room temperature overnight to release all of the electrolytes. The conductivity was measured again for the 100% leakage measurement (EL_T). The relative leakage (EL_R) was calculated as (EL_I) /(EL_T)x100. Three replicate measurements were performed for each plant.

After measurements of non-acclimated plants were completed, the plants were cold acclimated in a 4° C room with 8 hours of soft-white light per day for 10 weeks. For the

post-acclimation testing, the methodology was identical other than the cooling rate which was increased to 4° C h^{-1} and the samples were removed every 5 degrees from -5 to -30° C. This range was chosen because preliminary studies showed that cold acclimated plants suffered very little damage at the temperatures used for non-acclimated plants. It was also determined that it was unnecessary to pour out half of the leachate prior to autoclaving so the leachate and the leaves were autoclaved in one tube for all remaining electrolyte leakage assays.

First year A. kamchatica plants—

Measurements of electrolyte leakage were begun when all of the plants were at least 45 days old. Because of germination rate differences and the time required to perform the electrolyte leakage assay on 426 plants, some individuals were older than 45 days when the assays were performed. Up to 3 individuals were tested for each family (northern population: 57 families, n=155; mid-latitude: 59 families, n=178; southern: 42 families, n=93). While a few plants started to flower before their initial measurements, none were allowed to set fruit and there was not a difference in the electrolyte leakage values between plants that were and were not flowering at time of measurement (likelihood ratio test of linear mixed effect models with p-value calculated by the parametric bootstrapping method of Faraway (2002): D=0.909, p=0.342).

Second year A. kamchatica plants—

After the electrolyte leakage assay was completed, plants were returned to the greenhouse and allowed to re-acclimate to summer conditions. During this time, plants were allowed to flower and set fruit. Fruits were removed before reaching maturity to prevent new seedlings from germinating in the conetainers. After the plants were re-acclimated to greenhouse conditions for 5 months, a haphazard sample of 1 plant from each of 24 families from each population was selected for further electrolyte leakage testing. Of the 24 plants from each population that were selected, 12 were immediately tested for electrolyte leakage; these were not cold acclimated plants. The remaining 12 plants from each population was immediately cold acclimated in a 4° C room with 8 hours of soft-white florescent light per day for 4 weeks and then were used for the cold

acclimated electrolyte leakage trial. Although this treatment does not precisely mimic fall cold hardening in any natural population, exposure to low but non-freezing temperatures (2-6° C) is the standard method of inducing cold acclimation for freeze tolerance studies (XIN and BROWSE, 2000).

The methodology was the same for testing electrolyte leakage in first and second year plants except for a few minor changes. In second year plants, non-acclimated leakage samples were taken out of the cold bath at 0, -2, -6, -10, -14, and -18° C; acclimated leakage samples were taken out at 0, -4, -11, -18, -25, -32 and -35° C.

Supercooling assay—

Freezing events were detected as a measurable release of heat, or an exothermic event, for individual detached leaves and were recorded using LogXR software with a programmable XR5-SE-M data logger from Pace Scientific Inc. (Mooresville, NC, USA). For each supercooling assay, an expanded leaf was removed from 10 *A. kamchatica* 2nd year plants from each population and from one *A. thaliana* plant from each accession. All individuals tested for supercooling had already been tested for electrolyte leakage. Each leaf was placed in the bottom of a 10x13 mm glass tube. A PT-907 temperature probe from Pace Scientific Inc. was placed against the surface of each leaf and secured using foam. The tubes were placed into racks and immersed in a NesLab programmable circulating ethanol bath. The tubes were equilibrated at 0° C for 30 minutes and cooled at a rate of 4° C h^{-1} down -35° C. Temperature was recorded every second. The supercooling point was the lowest sub-zero temperature reached before the exothermic release (Fig 1.3). Three replicate leaves were tested for each plant.

Antifreeze protein assay—

To determine if *A. kamchatica* shows evidence of antifreeze protein activity, we used a solute assay to detect differences in the freeze point (FP) and melting point (MP) of cellular fluids, which is an indication of thermal hysteresis, whereby antifreeze proteins are lowering the FP below that of the MP in a non-colligative manner (URRUTIA *et al.*, 1992). Three levels of acclimation were assayed: overwintering plants (n=3) were dug up

from an outdoor garden plot located at the University of Alaska Fairbanks' Institute of Arctic Biology greenhouse (64°51' N, 147°50' W, elevation of 177 m) during mid-February; greenhouse plants (summer acclimated, n=4); and cold acclimated plants that were acclimated in a 4° C room with 8 hours of soft-white florescent light per day for 4 weeks (n=4). A leaf was removed from each plant and the petiole was squeezed in a downward direction from the leaf blade to the base and the resulting fluids were sucked into a capillary tube. The drop of fluid was put into an oil suspension. The drop was quickly frozen using an Otago Osmometer (Dunedin, NZ). A microscope was used to visualize the frozen drop. The temperature was minutely adjusted to isolate a single crystal of ice by slowly thawing the frozen drop. The melting (MP) and freezing point (FP) of this crystal was determined by adjusting the temperature by very small amounts.

Common garden—

A common garden with individuals from all three *A. kamchatica* populations was planted to determine if there were genetic differences among populations in overwinter survival, and to determine the relative fitness of plants from southern latitudes in a northern environment. The common garden was located outside of the University of Alaska Fairbanks' Institute of Arctic Biology greenhouse. This location is near the northern edge of the species range for *A. kamchatica* (Fig 1.1) and represents some of the coldest conditions this species naturally experiences.

For 2007-08, plants grown from greenhouse-collected seeds from 2 populations were planted in the garden. There were a total of 61 individuals from 12 families from the mid-latitude population and 63 individuals from 16 families from the southern population used in this garden. In 2008-09, plants grown wild-collected seeds from each of the 3 populations were planted in the garden. A total of 45 individuals from 18 families were from the northern population, 169 individuals from 54 families were from the mid-latitude population and 13 individuals from 7 families were from the southern population.

The seeds were planted in 15 cm pots in the greenhouse in mid-July. Approximately thirty days after germination, seedlings were acclimated to outdoor conditions, first in the shade then in full sun over a 7-day period in late August, and were placed into the ground

with full sun exposure at the end of August. Plants were given supplemental water as necessary during the summer months. During fall, the need for watering decreased and watering was stopped completely after the first snowfall. Plants remained snow covered and undisturbed throughout the winter. Leaves from the previous season persisted with a stressed appearance so over-winter survival was easily determined by the presence of new green leaves. Survival was scored in early May, approximately 2 weeks after snow melted from the plants. When all plants were finished flowering (late July) the final fruit number was counted for each plant. Herbivory damage was not noticeable in the 2007 garden, but in the 2008-09 garden, widespread herbivory damage, apparently from grasshoppers, was detected, so the pots and plants were enclosed in bridal veil until after the first snowfall. In natural populations herbivory damage, especially that from grasshoppers, is uncommon (personal observation).

Electrolyte leakage analysis—

Our methodology for estimating the LT50 for each population used relative electrolyte leakage as the dependent variable and the independent variables were family, individual, and date of EL measurement as the random effects and population as the fixed effect. In this model, these independent variables were allowed to have linear effects on the two parameters (the inflection point and scale parameter) of the logistic function. Family structure within populations and the sigmoidal nature of the data made it necessary for us to use a non-linear mixed-effects model to analyze the electrolyte leakage data. To estimate the point at which 50% of tissue would be damaged (LT50), we fit the sigmoidal curve in which the relative leakage EL_R at the temperature T follows a two-parameter logistic model:

$$EL_R = 1 - 1 / \{1 + \exp[-(T - d) / s\},$$

where the inflection point, d, gives the LT50 estimate, and s is a scale parameter. With this method, the lower asymptote of the curve approaches 0 at temperatures that are too warm to cause damage to the leaf tissue, the upper asymptote approaches temperatures at which the most cells would be lysed. The sigmoidal curve fitting method has been widely used in cold tolerance reporting, and various methods are used to fit the

data to the curve (HANNAH *et al.,* 2006; HAO *et al.,* 2009; LE *et al.,* 2008). We used the logistic function in the R statistical package (R DEVELOPMENT TEAM, 2011).

For between species comparisons, our independent variables were species and acclimation treatment as fixed effects and population, family, individual, and date of EL measurement as random effects. We used *nlme* package (PINHEIRO and BATES, 2000) implemented in R Statistical Environment (R DEVELOPMENT TEAM, 2011). We first conducted a model selection (FARAWAY, 2006) to include the relevant random effects by Akaike Information Criterion (AIC), following Pinheiro and Bates (2000).

We estimated the LT10 and LT90 values by fitting the data to a sigmoidal curve and then performed non-parametric bootstrapping for 1,000 iterations to determine at what temperatures the asymptote approached 10 and 90% damage and to estimate the confidence intervals for each estimated parameter (FARAWAY, 2002). In the bootstrap, each observation (the percent electrolyte leakage measurement) was the unit of resampling, and the total number of observations was constrained to match the data. Briefly, non-linear mixed-effects models, containing the relevant independent variables as described above, were fitted to each bootstrapped data set, and confidence intervals were calculated from the distribution of estimated parameters.

In order to test the significance of fixed effects, i.e., whether acclimation and/or species influenced the shape of the electrolyte leakage response curve, we performed likelihood ratio tests to compare models with and without a fixed effect (including the interaction of species and acclimation effects). The test statistic reported for the likelihood ratio tests is D, where D is twice the difference in the log-likelihoods of two models: D = -2 ln (likelihood for a simpler model / likelihood for a model with more parameters).

We checked for normality and homogeneity of errors by inspections of plots of residuals against fitted values and Quantile-Quantile (QQ) plots. If the assumptions were violated, we attempted to fit extended non-linear mixed-effect model, where appropriate variance functions can be used to model heteroscedasticity of within group errors (Chapter 8, PINHEIRO and BATES, 2000). The variance function we used was

'varPower(fixed=0.5, form= ~ fitted(.) - fitted(.)^2))'. However, if this correction failed to converge due to added complexity, we were forced to use the original models without the correction.

Supercooling analysis—

In order to accommodate the family structure within populations, we used a linear mixed-effects model to analyze supercooling data. The dependent variable was supercooling point. For species level comparisons, the fixed effects were species and cold acclimation treatment while the random effects were population, individual, and date of trial. For population level comparisons within a single species, the fixed effects were population and acclimation while the random effects were individual and date of trial. We determined 95% confidence intervals by bootstrapping the model with the lowest Akaike Information Criterion (AIC) score from the linear mixed modeling for 5,000 iterations.

A variety of methods have been used to compare supercooling and LT50 values: Bravo *et al.* (2001) used one-way ANOVA to compare the supercooling point and LT50 for Antarctic angiosperms, while Sklenar (2010) and Sierra-Almeida (2009) used a t-test to compare these values in Andean plants. However, because the supercooling point and LT50 values are derived from very different types of data, and will necessarily have a very different variance structure, we chose to simply compare means and 95% confidence intervals (Fig 1.4).

Antifreeze protein analysis—

A linear mixed-effects model was used to determine if there was a difference in melting and freezing point among acclimation treatments. The temperature was the dependent variable and the independent variables were acclimation treatment and MP vs FP as the two fixed effects, and leaf (MP and FP were measured for each leaf) and individual plant as the random effects. The dependent variable was transformed with Box-Cox transformation (Box and Cox, 1964; Fox and Weisberg, 2011).

Common garden analysis—

ANOVA was used to detect differences in fruit number among populations. Pearson's Chi-square test was used to determine if there were differences in over-winter survival. The common garden experiment does not directly determine if exposure to cold temperatures caused differences in fruit production or over-winter survival because many factors (such as photoperiod, snow depth, plant size) all would have an affect on plant overall fitness and survival, however it does allow us to look for differences among populations after exposure to winter in Fairbanks, AK.

All statistical analysis was completed in the R Statistical Environment (R DEVELOPMENT TEAM, 2011).

RESULTS

Differences between species—

To determine whether there were differences in cold tolerance between *A. kamchatica* and *A. thaliana*, we used electrolyte leakage to estimate the temperature at which 50% of cells were lysed by freeze damage (LT50). In both non-acclimated and acclimated plants, LT50 was considerably lower for *A. kamchatica* than *A. thaliana* (Fig 1.4, non-acclimated: D=8.77, df=8, p = 0.003; acclimated: D=16.92, df=7, p <0.001). Further, *A. kamchatica* had a much higher cold acclimation capacity than *A. thaliana* as seen by the difference in LT50 before and after cold acclimation (Fig 1.4), the acclimation process led to a 12.4° C decrease in LT50 values for *A. kamchatica* leaves and only a 1.6° C decrease for *A. thaliana* leaves.

Thermal analysis was used to measure the temperature at which freezing was initiated in the absence of external ice nucleation (the supercooling point). A single exothermic event was detected for each of the plants measured (Fig 1.3). *A. kamchatica* supercooled significantly more than *A. thaliana* (Fig. 1.4, D=13.75, df=1, p=0.002). Surprisingly, acclimated *A. kamchatica* plants supercooled slightly less than non-acclimated plants

(D=9.381, df=2, p=0.005), but in *A. thaliana,* there was no significant difference in the supercooling point before and after acclimation (Fig 1.4).

A comparison between LT50 and the supercooling point can be used to determine whether plants are using a freeze tolerance or freeze avoidance strategy. In both non-acclimated and acclimated *A. thaliana*, these values were similar, with overlapping 95% confidence intervals, suggesting that this species may be able to avoid freezing if they are not externally nucleated. However, *A. kamchatica* appears to be quite different. In non-acclimated leaves, the supercooling point is lower than the LT50, but in acclimated leaves, the LT50 is much lower. This suggests that during the growing season plants may be able to supercool to avoid freezing during a short cold spell, but plants become freeze tolerant when they are acclimated for winter.

Antifreeze protein assay—

To determine if *A. kamchatica* showed evidence of antifreeze protein activity, we used a solute test to look for differences between the melting (MP) and freezing point (FP) of cellular fluids, which would indicate thermal hysteresis (URRUTIA *et al.,* 1992). While there was a difference in FP and MP for all acclimation treatments (Table 1.1, D=27.756, df=1, $p < 0.0002$), the level of thermal hysteresis was an order of magnitude smaller than those found in antifreeze protein expressing plants (URRUTIA *et al.,* 1992). Additionally, overall melting and freezing points differed between the acclimation treatments (D=25.345, df=2, $p < 0.0002$). While plants that express antifreeze proteins are expected to show thermal hysteresis after acclimation (GRIFFITH and YAISH, 2004; URRUTIA *et al.,* 1992), in *A. kamchatica* there was not a change in thermal hysteresis after acclimation (D=7.63, df=3, p=0.0676), The extremely low amount of thermal hysteresis and the lack of acclimation effect indicates that there was not antifreeze protein activity in *A. kamchatica.*

Latitudinal differences among populations of A. kamchatica—

To investigate differences among populations of *A. kamchatica*, and determine if northern plants are more cold tolerant, we measured supercooling and electrolyte leakage

in plants from three populations of *A. kamchatica* representing the extreme edges of the North American latitudinal range of this species. In both non-acclimated and acclimated plants, the population with the lowest LT50 value was the mid-latitude population (Fig 1.5). There was also a difference in supercooling among the populations (D=6.33, df=2, p=0.045, Fig 1.5) with the northern population having a slightly higher capacity. The same pattern of differences among populations was observed in plants from both years. The average air temperatures at the southern and mid-latitude population origins are very similar and remain near freezing, however temperatures are far more extreme at the northern population (Fig 1.6).

Common garden—

We estimated fitness of *A. kamchatica* plants from all three populations in a single common garden by measuring overwinter survival and reproduction. *A. kamchatica*'s North American range extends from 49°49' N to 65°42' N latitude. Fairbanks is located at 64°5' N, so our common garden was located at the northern extreme of the distribution, and tested over-winter survival at possibly the most extreme cold conditions populations would experience in nature. Survival rates in the common garden experiments were high and for both years there was no significant difference among populations (Fig 1.7, 2007-08: χ^2=0.626, p=0.429, df=1; 2008-09: χ^2=1.472, p=0.479, df=2, n.s.). In 2007-08, 97% of the mid-latitude and 92% of the southern plants survived. In the 2008-09 garden, survival was again high for all populations; 89% of northern, 94% of mid-latitude and 92% of southern plants survived. We also compared fruit production among populations after over-wintering in Fairbanks, AK. Without measuring fruit production with and without cold stress, it is not possible to directly determine the magnitude of the affect that exposure to winter conditions had on fruit production but the mid-latitude plants produced the most fruits in both years (Fig 1.8, 2007-08: p=0.0003, df=1; 2008-09: p=0.0007, df=2). Thus the overall fitness of the mid-latitude seems to be highest in our garden, even though the garden was located at a much higher latitude than the population originated from.

DISCUSSION

Differences between species—

The study of cold tolerance in plants may lead to a better understanding of adaptation at high latitudes. Most climate change scenarios predict that climate change will be more extreme at high latitudes (BOKHORST *et al.,* 2010), therefore a better understanding of the cost of cold tolerance in plants is an important factor for future modeling efforts. Already, global warming along with other ecological stresses such as invasive species and habitat fragmentation has modified the distribution of many species (PARMESAN *et al.,* 2005). Yet species differ in their sensitivity to changes in climate, and changes in temperature regimes may lead to wide-spread environmental changes such as altered precipitation and herbivory (VALLADARES *et al.,* 2007), making it difficult to predict how species will respond to changes in climate.

Cold tolerance has been well studied in *A. thaliana*, however this species is not found in the far north where more extreme cold tolerance is required for survival. *A. thaliana* has a wider climatic and geographic distribution than *A. kamchatica*; however the range of *A. kamchatica* extends much further north (HOFFMANN, 2005) and thus this species is expected to be adapted to more extreme cold. *A. kamchatica* was capable of tolerating much colder temperatures than *A. thaliana* both before and after acclimation. Tolerance of extreme cold may be quite different from tolerance to milder cold temperatures. With its higher acclimation capacity and deeper cold tolerance, *A. kamchatica* cold tolerance research provides a useful complement to research in *A. thaliana*.

Over-winter strategy—

By measuring both the supercooling capacity and quantifying LT50, we were able to determine the over-winter strategies of *A. kamchatica* and *A. thaliana.* It appears that *A. kamchatica* and *A. thaliana* species employ different strategies for over-winter survival. After *A. kamchatica* plants are cold acclimated, they appear to tolerate freezing, because the temperature at which 50% freeze damage occurred was much colder than the supercooling point. In comparison *A. thaliana* the LT50 was at or near the supercooling

point; and thus, *A. thaliana* plants avoided freezing. Neither species supercooled more after acclimation than before; thus, supercooling does not appear to be part of the cold acclimation mechanism, but is a constitutive trait.

Freezing avoidance, the strategy found in *A. thaliana,* may not be effective against extreme or prolonged cold temperatures (SIERRA-ALMEIDA *et al.,* 2009); therefore, freeze tolerance, the strategy we found in *A. kamchatica,* is thought to be the main mechanism for plant over-winter survival (PUHAKAINEN *et al.,* 2004; SAKAI and LARCHER, 1987). Though the freeze tolerance strategy is more common, there are plant species that successfully employ freeze avoidant strategies for over-winter survival. For example, the Antarctic plant *Colobanthus quitensis* has a moderate supercooling point of -9.4° C when cold acclimated for 21 days, however its LT50 is only -5.8° C, indicating this species avoids freezing by supercooling (BRAVO and GRIFFITH, 2005; BRAVO *et al.,* 2001).

While most freeze tolerating plants limit supercooling (SAKAI and LARCHER, 1987), some Hawaiian high altitude plants were found to supercool to -5 or -9° C, and had measured LT50 values that were several degrees below this, suggesting that these plants tolerate freezing, but that a period of supercooling may occur prior to freezing without resulting in severe damage from ice nucleation of supercooled tissues (LIPP *et al.,* 1994). The same process may occur in *A. kamchatica* because the acclimated LT50 value was much colder than the supercooling point; however the supercooling point was below -10° C. Alternatively, because *A. kamchatica* is a small rosette plant that is covered by snow in winter, snow likely initiates freezing in the leaves (PEARCE, 2001) at temperatures above the supercooling point.

There is a trade-off between plant growth and freeze resistance so during periods of high growth, freeze resistance is lowest (LARCHER *et al.,* 2010). Therefore short term frost events during the growing season can cause massive economical losses (KREYLING, 2010). Nevertheless, some plants have a relatively low supercooling point even when non-acclimated. For example, in non-acclimated *A. kamchatica* plants the supercooling point was colder than the LT50, indicating that during the growing season this species would be able to use supercooling to avoid freezing. However, *A. kamchatica* appears to

develop the ability to tolerate freezing during the cold acclimation process. Seasonal changes in the freeze resistance mechanism were also found in some Andean plants; however this phenomenon has rarely previously been reported or studied (SIERRA-ALMEIDA *et al.,* 2009).

The overall trends for freeze damage were the same in 1st and 2nd year *A. kamchatica* plants, even with a shorter acclimation time in the 2nd year plants. In addition, *A. kamchatica* plants appeared to be hardier after the first summer/winter cycle (Fig 1.5). This trend has been found in other species, for example one study found increased fitness in *A. thaliana* following cold acclimation treatment (ZHEN *et al.,* 2011). In *Rhododendron*, leaf freezing tolerance increased by approximately 5-6° C during the first 2-3 years for plants from most populations tested (LIM *et al.,* 1998). This study predicted that maximum cold hardiness would not be attained until the plants reached reproductive maturity (LIM *et al.,* 1998). It might also be expected in nature that plants during their second winter would have less freeze damage because older plants are often larger and have more resources at their disposal.

While there was not evidence of antifreeze proteins in *A. kamchatica*, these proteins have been found in many other freeze tolerating plants (GRIFFITH and YAISH, 2004). Much remains to be learned about plant antifreeze protein activity. In winter cereals for example, six antifreeze proteins have been identified; and although the exact function of these proteins remains to be discovered, it has been shown that they can depress the freezing point of winter rye as much as 1.26° C (GRIFFITH *et al.,* 2005). In plants, antifreeze proteins correspond to classes of pathogenesis-related proteins which are associated with disease resistance in plants (GRIFFITH and EWART, 1995). A promising area of crop cold tolerance research is in the creation of transgenic plants with fish or insect antifreeze proteins (HUANG *et al.,* 2002). Thus far, transgenic lines expressing insect antifreeze proteins have been more successful for increased thermal hysteresis activity than those expressing fish proteins (HUANG *et al.,* 2002). While antifreeze proteins have little effect on the LT50, they may reduce crop losses to light frosts by slowing the freezing process (GRIFFITH and YAISH, 2004).

Latitudinal differences among populations of A. kamchatica—

Winter air temperatures are more extreme in the northern population than the other populations (Fig 1.6); therefore we expected that the northern population would have the coldest supercooling point and LT50. However, the mid-latitude population had the lowest LT50. It is possible that the mid-latitude plants actually experience the most cold since the warmer temperatures (Fig 1.6) could allow the insulative layer of snow to melt, exposing plants to subsequent cold snaps. Because the data loggers that we placed above and below the snow in natural populations were vandalized, we do not know the actual temperatures experienced by plants in winter. However, both the southern and mid-latitude plants grow in mountainous regions that receive copious snowfall, and that this snow takes a long time to melt in spring (personal observation). In the town Whittier (located approximately 10 km from the mid-latitude population), snow records from 1950-2011 show the average snow depth in March is 1.17 m and the annual average snowfall is over 6.5 m (CENTER, 2012). This depth of snow means plants in this population are most likely buffered from the freeze-thaw temperatures for much of the winter.

It also is possible that other factors, such as overall vigor of plants from different populations could have influenced the LT50. The mid-latitude population is less likely to suffer from expression of genetic load because it is several orders of magnitude larger than the other populations, it is in a less ephemeral habitat (glacial moraine, rather than streamside), and it is located in the center of the species' North American range (BRIDLE and VINES, 2007; KIRKPATRICK and BARTON, 1997; LYNCH *et al.,* 1995). Mid-latitude plants produced the most fruits in our common garden, and this population generally appears to be more vigorous (personal observation). Additionally, we have data to suggest that genetic diversity, at least for LT50, is higher in the mid-latitude population than in other populations (Appendix 1).

The lack of differences in over-winter survival in our common garden along with the similarity in LT50 between the populations indicates that there hasn't been a loss in cold tolerance for the southern population. The cost of cold tolerance may not be high enough

to drive selection against this trait; or the population expansion could have been so recent that there hasn't been time for selection against this trait, even though plants from southern populations experience far milder winters, with the temperatures rarely dropping below freezing. In *A. thaliana*, studies were unable to establish an allocation cost for cold acclimation and, similar to our results, cold acclimation capacity did not appear to be selected against in southern environments (ZHEN *et al.*, 2011).

Microclimate undoubtedly also plays an important role in over-winter survival for both *A. kamchatica* and *A. thaliana*. The amount of freeze damage plants experience is not just determined by temperature. A variety of other factors, including water content, leaf size, plant height, and leaf litter all influence a plant's ability to resist freezing temperatures (SIERRA-ALMEIDA *et al.*, 2010). A layer of snow can protect plants from photoinhibition and freeze induced dehydration due to exposure (LARCHER *et al.*, 2010). As our data show (Fig. 1.2), snow may be especially important during extreme cold events because as little as 30 cm of snow can act as an effective insulator to decouple soil and air temperature (BILLINGS and BLISS, 1959; KREYLING, 2010). Snow cover also provides protection during spring and fall when daytime temperatures often rise well above freezing but nighttime temperatures can still plummet well below 0° C (TAULAVUORI *et al.*, 2011). *A. thaliana* is found in many places where snow is rare or absent meaning overwintering plants could be exposed to cold temperatures and other winter stresses. In contrast, all of the *A. kamchatica* populations we tested are likely to be insulated by snow in winter and would be unlikely to experience the -20 to -30° C air temperatures found in nature. During summer, plants don't have the protection offered by a layer of snow, and in the growing season freeze resistance is lowest; thus having sufficient non-acclimated cold tolerance is important for surviving sudden freeze events. Among *A. kamchatica* populations from this study, the potential for a summer frost is much more likely in the Alaskan populations than the more southerly Canadian population, yet all populations were similar in non-acclimated cold tolerance. Predicted changes in climate may lead to increased variability in temperature and precipitation year-round. The potential for abnormal winter warming events and more extreme short-

term cold events is likely in many climate change projections (BERTRAND and CASTONGUAY, 2003). There have already been changes in the extent of annual snow cover over the last 70 years and this trend is predicted to continue (KREYLING, 2010). This could lead to changes in the types of damage that plants experience and the balance between the growing season and preparedness for over-winter survival may become more challenging in the future (BERTRAND and CASTONGUAY, 2003).

Summary—

It might be expected that closely related species would have similar over-winter strategies, especially because their range distributions overlap in nature. This was not supported by our findings. *A. kamchatica* tolerates freezing for over-winter survival but switches strategy to avoid freezing during the growing season. In contrast, *A. thaliana* supercools to avoid freezing year-round and is much less tolerant to cold than *A. kamchatica.* Members of the *Arabidopsis* genus occupy diverse climates and habitats throughout the Northern Hemisphere (HOFFMANN, 2005) and among species there are differences in cold tolerance (Armstrong *et al.*, in prep) but supercooling has not previously been measured in *Arabidopsis* species other than *A. thaliana* to our knowledge. With *A. kamchatica* and *A. thaliana* having different cold tolerance strategies but a shared evolutionary history, it is unknown when in the *Arabidopsis* lineage freeze tolerance arose and how this has influenced current species distributions. More detailed sampling of both the LT50 and supercooling from other *Arabidopsis* species with known phylogenies is needed to uncover the evolution of the freeze tolerance trait within this genus.

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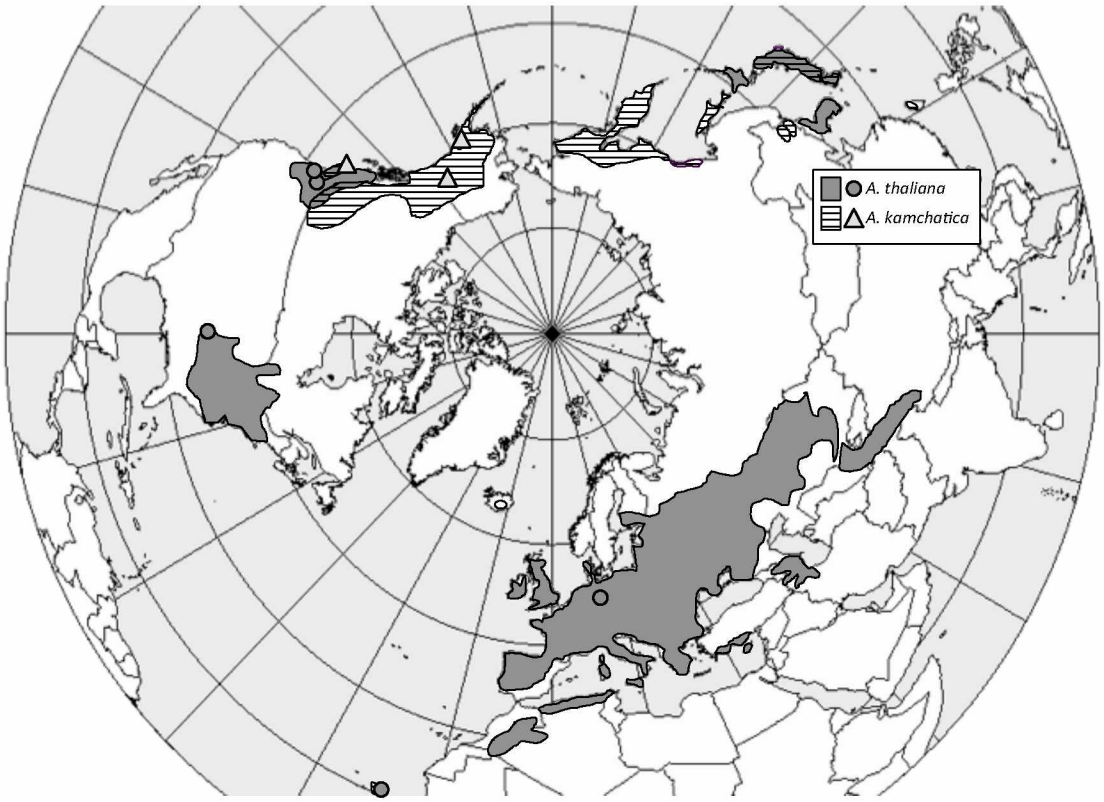
Table 1 Thermal hysteresis of non-acclimated (n=4), 6-week 4° C acclimated (n=4), and overwintering outdoor (n=2) *A. kamchatica* plants. There were no statistical differences among acclimation groups. Melting and freezing points were different from each other but the level of thermal hysteresis is not biologically relevant. *Mean±std.dev*

Acclimation state	Melting Point (°C)	Freezing Point (°C)	Thermal hysteresis
Non-acclimated	-0.48±0.146	-0.50±0.148	0.02
6-week 4 °C acclimated	-1.53±0.288	-1.56±0.288	0.031
February outdoor plants	-1.99±0.572	-2.01±0.949	0.022



http://tao-dnd.blogspot.com/2011/09/projection.html

Figure 1.1 A map of the *A. thaliana* (grey fill, circles) and *A. kamchatica* (black stripes, triangles) populations used for this study and the range of each species. Map modified from Hoffmann, 2005.

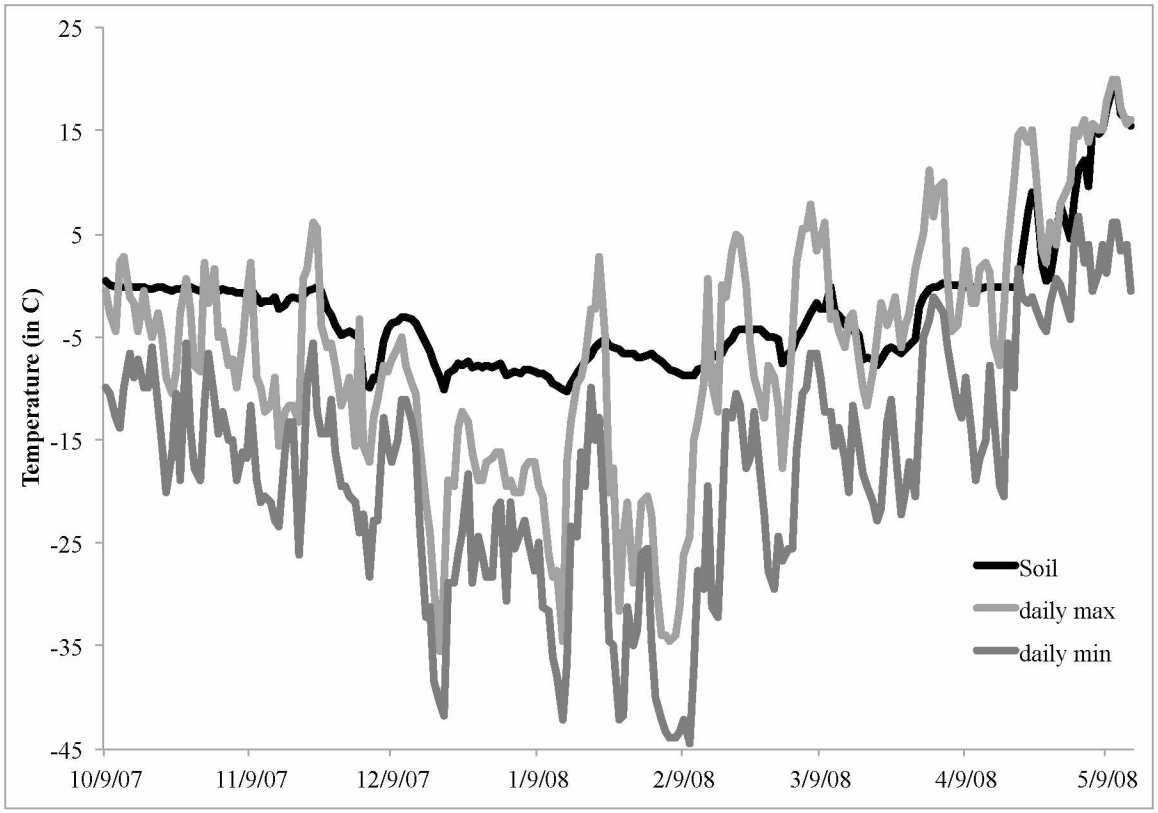


Figure 1.2 Soil surface temperatures below the snow (black line) and maximum (light gray line) and minimum air temperatures (dark grey line) in Fairbanks, AK from October 2007 to May 2008. Soil temperature data were recorded with a Hobo U23 Pro v2 data logger (Cape Cod, Massachusetts, USA). Air temperature data were obtained from http://climate.gi.alaska.edu/.

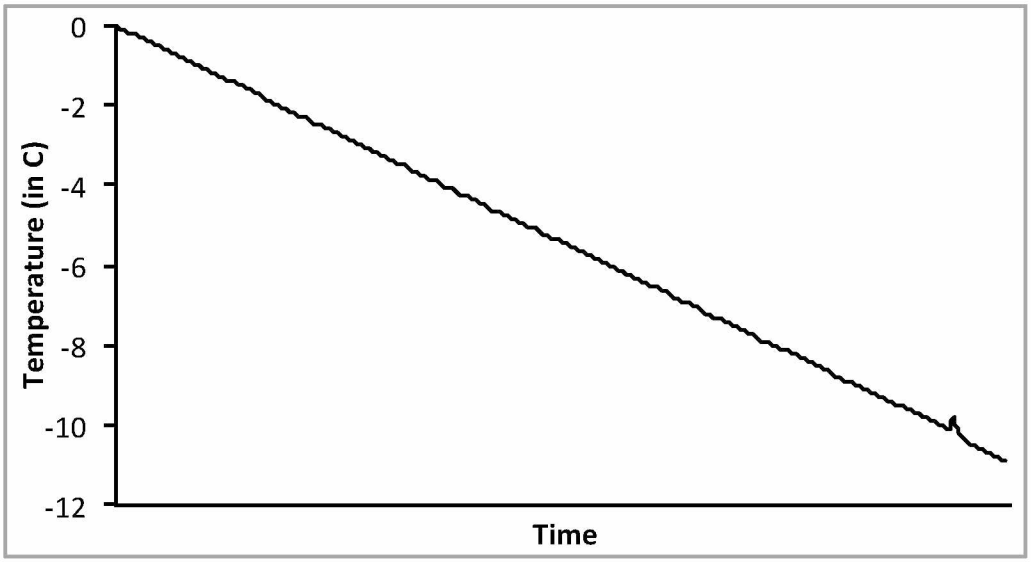


Figure 1.3 An example of supercooling data. A thermocouple measures the leaf surface temperature during cooling. In this example, heat is measured as an exotherm when water in the leaf freezes at the supercooling point, -10° C.

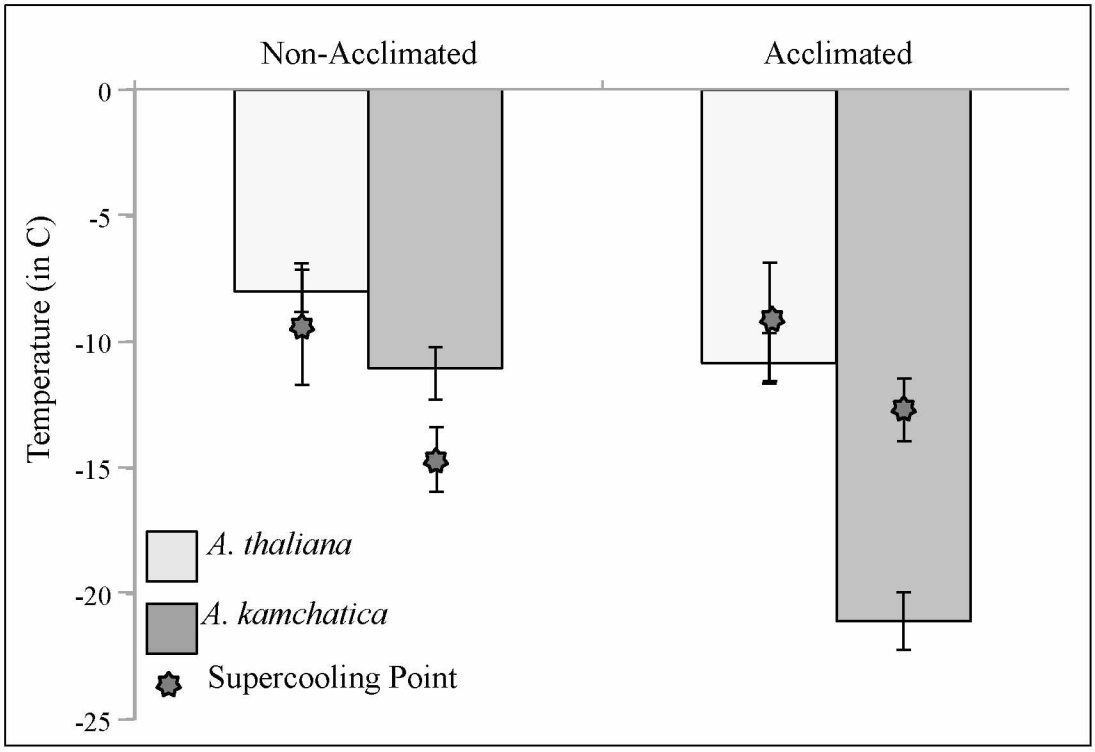


Figure 1.4 Mean LT50 of *A. kamchatica* (dark bars) and *A. thaliana* (light bars) before and after acclimation. The mean supercooling point is denoted by a star. Error bars represent 95% confidence intervals around the means.

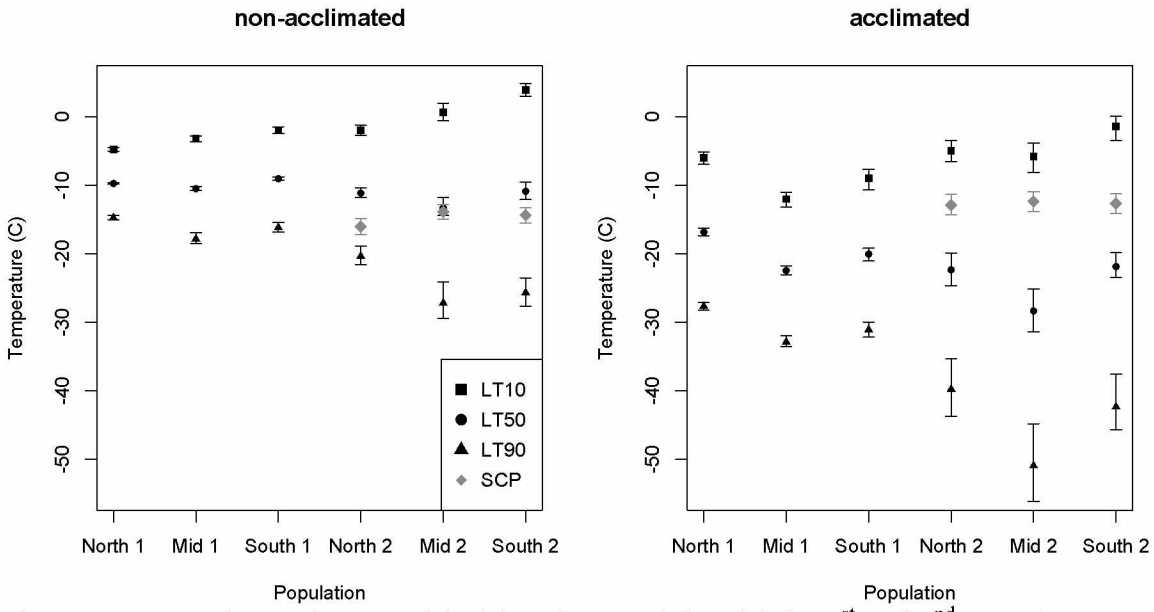


Figure 1.5 LT10 (square), LT50 (circle) and LT90 (triangle) for 1st and 2nd year *A. kamchatica* plants from the northern, mid-latitude and southern populations. The year and location of each population is shown on the x-axis (i.e. North-1 denotes 1st year plants from the northern population). Supercooling values for 2nd year plants are also shown (gray diamond). Error bars represent 95% confidence intervals around the means.

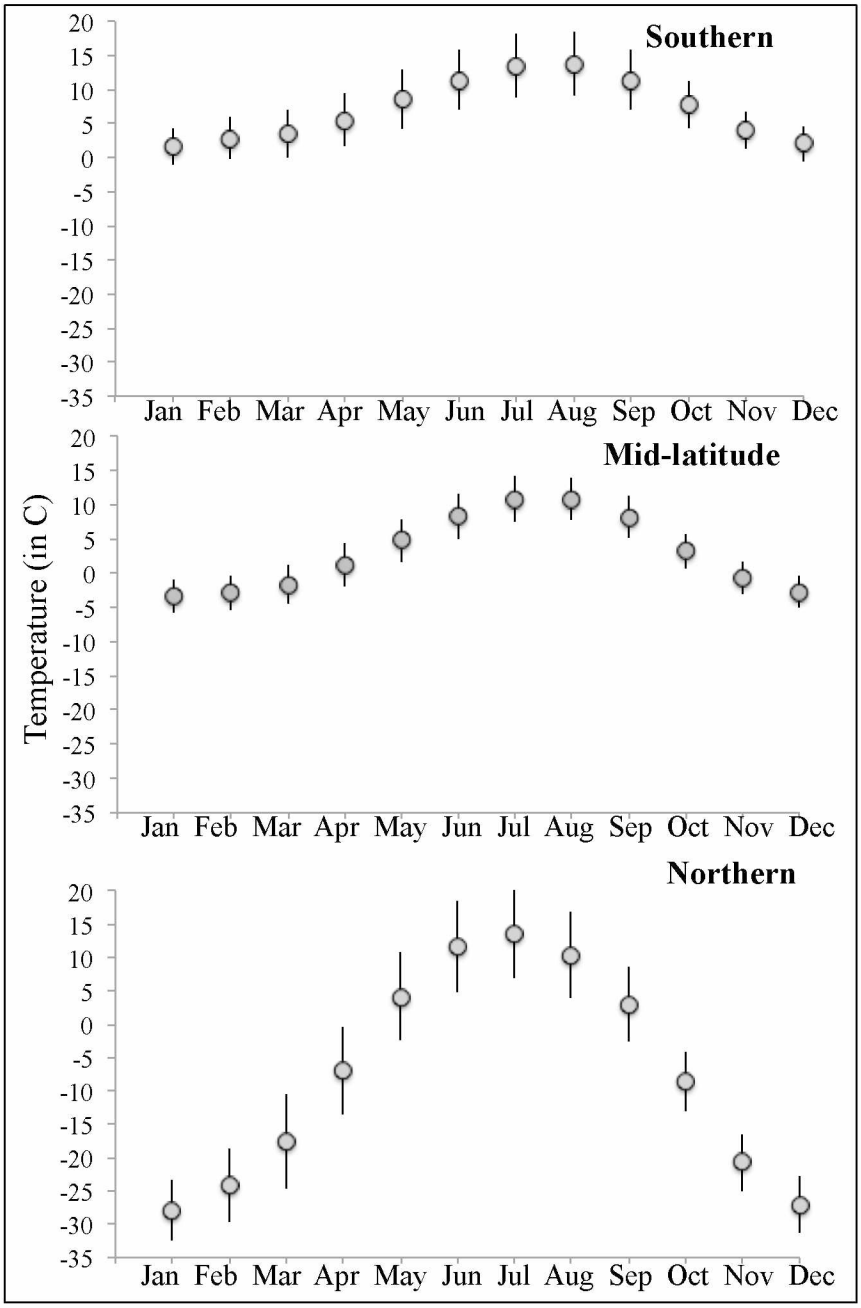


Figure 1.6 Average monthly air temperatures (°C) from the southern (Strathcona Park, British Columbia, Canada), mid-latitude (Portage Glacier, AK, USA), and the northern (Ptarmigan Creek, AK, USA) *A. kamchatica* populations of origin. Bars indicate the average maximum and minimum temperatures for each month. Air temperature data were obtained from ArcGIS using methods described in Steets *et al.*, 2010.

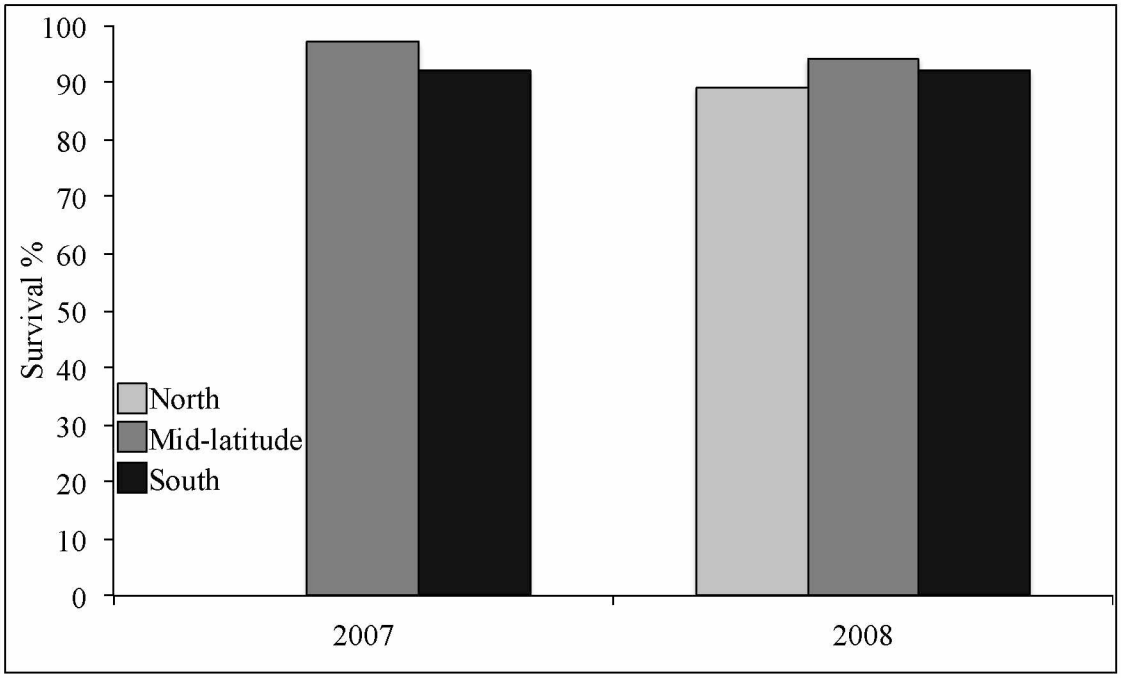


Figure 1.7 Over-winter survival rate for each population of *A. kamchatica* from the common garden in 2007 and 2008.

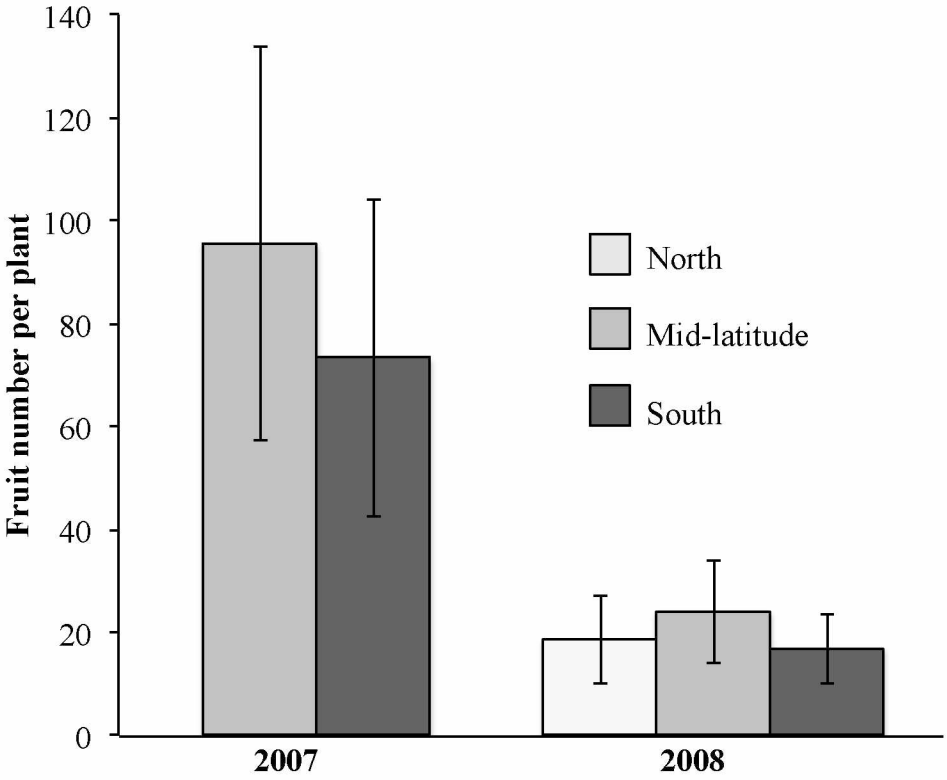


Figure 1.8 Average number of fruits for each *A. kamchatica* population from common garden plants in 2007 and 2008. Values are means ±SD.

APPENDIX 1

Genetic variation in cold tolerance within a population is necessary for adaptation to changes in climate (SAKAI and LARCHER, 1987). We used the data from Chapter 1 to investigate genetic variance in cold tolerance, and differences among populations in genetic variance for cold tolerance. However, these data are only included in an appendix because they are not fully developed enough to be an entire chapter, but are slightly too divergent to be included in the other chapters.

We compared the amount of genetic variation for cold tolerance among populations of *A. kamchatica* in the two parameters describing the logistic function (the LT50 estimate, *d*, and the scale parameter, *s*). The analyses were similar to estimation of the LT50 for each population as described in Chapter 1. However, for this purpose, we fitted the logistic function to each population since we would not want to assume that all populations had a homogeneous distribution for the family random effect. We used relative electrolyte leakage as the dependent variable and the independent variables were family and individual as the random effects. Then, we predicted the random effect for each family with "best linear unbiased prediction" (BLUP) under the selected model. Although a maternal family, collected from the field, could be a mixture of full- and half-sibs, we considered the BLUPs to be the approximate breeding values and the amount of variation among the breeding values represents an estimate of genetic variation within populations. We performed a non-parametric Levene's test to determine if the amount of genetic variation was equal among populations. We then performed post-hoc pair-wise comparisons between populations to see which populations are different in terms of the amount of genetic variation with the Bonferoni correction.

All populations contained genetic variation in LT50 both before and after cold acclimation (Fig A.1). The amount of genetic variation in LT50 differed among populations in both non-acclimated and acclimated plants (non-acclimated: $F_{2,158}=52.752$, $p < 10^{-15}$ acclimated: 1 $F_{2,157}=15.025$, $p < 10^{-5}$; Fig A.1). For the non-acclimated plants, post-hoc comparison revealed that all populations differed from each other, and the mid-latitude population had the highest amount of genetic variation among families before

acclimation, and the northern population had the lowest (mid-latitude vs northern: $F_{1,115}$= 108.84, P<10^{-15}, mid-latitude vs southern: $F_{1,101}$= 11.599, P<0.001, northern vs southern: $F_{1,100}$=57.585, p<10^{-10}). After acclimation, the mid-latitude and the southern populations did not differ, but they both had higher amounts of genetic variation than the northern population (mid-latitude vs northern: $F_{1,114}$= 46.317, P<10^{-9}, mid-latitude vs southern: $F_{1,101}$, NS, southern vs northern: $F_{1,99}$= 9.408 P<0.003).

The high genetic diversity in the mid-latitude population, and lower diversity at the edges of the range is consistent with theory regarding central and marginal populations (SEXTON *et al.*, 2009), and may suggest that the mid-latitude population will be more capable of adapting to climate change.

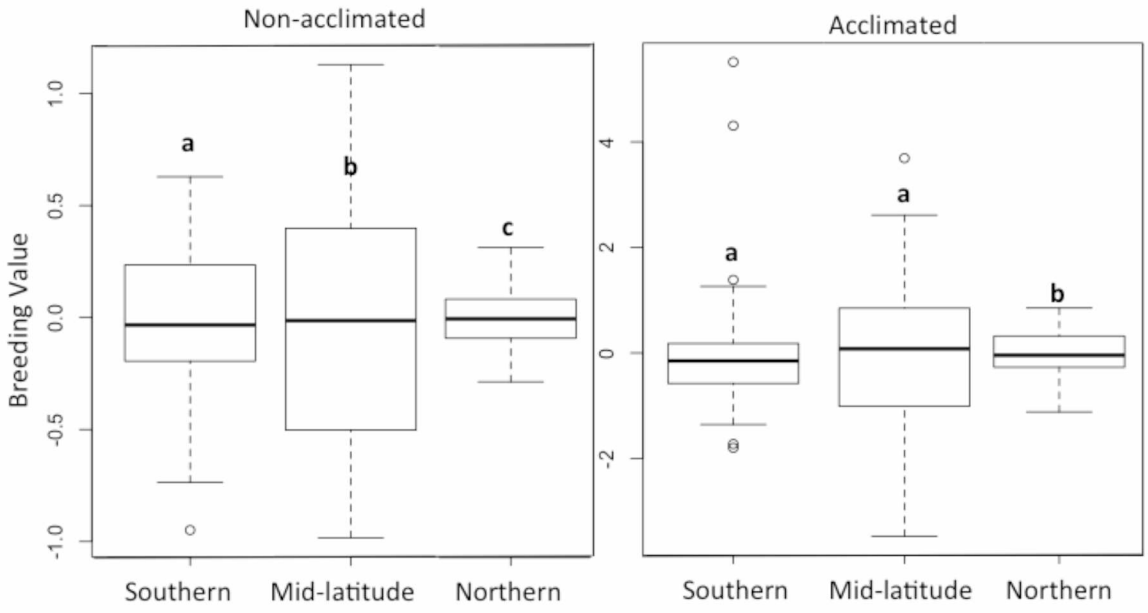


Figure A.1 Distribution of LT50 breeding values for each population. The dark line shows the median, the box shows the first and third quartiles, wiskers represent the minimum and maximum values excluding outliers, and circles show outliers (values >1.5x the upper quartile). Letters indicate significant differences among populations in the amount of variation in the breeding value, an index of genetic variance.

Chapter 2: Cold tolerance within and among *Arabidopsis* (Brassicaceae) taxa[2]

ABSTRACT

Premise of the study: Cold tolerance is an important distribution-limiting parameter for plant species, yet few studies have examined variation in cold tolerance extensively within and among closely related species. This is the first study to compare cold tolerance in several species of the genus *Arabidopsis*.

Methods: We assessed inter-specific variation in cold tolerance by measuring electrolyte leakage from detached leaves in five taxa of *Arabidopsis*. In four of the taxa, multiple populations were tested to examine latitudinal variation in cold tolerance.

Key results: The closely related *A. kamchatica, A. lyrata* subspecies *petraea,* and *A. lyrata* subspecies *lyrata* were more cold tolerant than *A. thaliana* and *A. halleri* subspecies *gemmifera* before and after cold acclimation. Cold tolerance increased after cold acclimation for all but *A. halleri* subspecies *gemmifera* and there was not a relationship between latitude of population origin and cold tolerance for the populations we tested.

Conclusions: Plants from all but one of the taxa of *Arabidopsis* we tested increased their cold tolerance after exposure to low, but non-freezing, temperatures. We found variability within and among taxa in cold tolerance after cold acclimation. Taxa with northern range distributions tended to be more cold tolerant than taxa that have southern range distributions however there was not a latitudinal cline within any of the individual taxa.

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[2]Armstrong, J., D. E. Wolf, N. Takebayashi. Cold tolerance in *Arabidopsis kamchatica*. Prepared for submission to American Journal of Botany.

INTRODUCTION

The ability to tolerate cold temperatures is considered to be one of the primary forces determining the range boundaries of many plant species (PITHER, 2003). Species distributions are also influenced by the ability of plants to increase their cold tolerance through a process called cold acclimation. Cold acclimation results from exposure to low, but non-freezing temperatures resulting in a cascade of biochemical, molecular, and physiological changes that allow the plant to increase cold tolerance (XIN and BROWSE, 2000). As sessile organisms, plants must be able to tolerate and adjust to the range of temperatures that occur in their environment both daily and seasonally.

The overwintering structures of plants growing at high latitudes may experience extreme cold, and northern plants often experience a broad range of temperatures throughout the year. The low diversity of plant species in the far north may in part be due to the inherent difficulties of adapting to the abiotic stress of life at high latitudes. Studies have demonstrated that latitudinal clines exist for many plant traits including the timing of growth cessation (PAGTER *et al.,* 2010), response to light wavelengths (STENOIEN *et al.,* 2002), and cold tolerance (ZHEN and UNGERER, 2008). Cold tolerance may have a physiological cost because cold acclimation is an inducible response; if there were no trade-offs, plants would always exhibit the same level of tolerance (XIN and BROWSE, 2000). If cold tolerance is physiologically expensive for plants to maintain, it could be expected that populations within the same species may exhibit latitudinal variation for this trait.

A. thaliana has been used as a model species for understanding the molecular and physiological underpinnings of cold tolerance in plants (HANNAH *et al.,* 2006; THOMASHOW, 1999; XIN and BROWSE, 2000). In *A. thaliana,* there is variation among accessions (populations) for non-acclimated and acclimated cold tolerance, as well as in the capacity to cold acclimate (HANNAH *et al.,* 2006). For *A. thaliana*, temperature is a distribution-limiting parameter (HOFFMANN, 2002) and populations from higher latitudes are more cold tolerant than populations from low latitudes (ZHEN and UNGERER, 2008).

While many studies have focused on *A. thaliana,* there has not been a within-genus comparison of cold tolerance in *Arabidopsis* to our knowledge. In addition, few studies have examined cold tolerance in populations at different latitudes from several members of a single genus (ADDO-BEDIAKO *et al.,* 2000; FRIEDMAN *et al.,* 2008; HANNAH *et al.,* 2006). Our study compares both inter- and intra-specific differences in cold tolerance in *Arabidopsis,* which allows us to determine if there is a latitudinal cline in cold tolerance within any of the taxa and to compare taxa to see how conserved cold tolerance is among related species.

Species from the *Arabidopsis* genus are distributed throughout the Northern Hemisphere in a wide variety of climates and habitats (HOFFMANN, 2005). The evolutionary history of this genus has been well studied and it is thought that *A. lyrata* and *A. halleri* diverged from *A. thaliana* approximately 5 million years ago, then split from each other 2 million years ago (AL-SHEHBAZ and O'KANE, 2002). Subsequently, *A. lyrata* diverged into *A. lyrata* subspecies *lyrata* (hereafter *A. l. lyrata*) and *A. lyrata* subspecies *petraea* (hereafter *A. l. petraea*) (AL-SHEHBAZ and O'KANE, 2002). *A. kamchatica* is an allotetraploid that originated from the hybridization of *A. halleri* and *A. l. petraea* (SHIMIZU-INATSUGI *et al.,* 2009).

To investigate differences in cold tolerance and acclimation capacity within and among species, we measured the freeze damage in excised leaves from five *Arabidopsis* species. The first goal of our study was to determine if *Arabidopsis* species differed in cold tolerance. We predicted that species with northern distributions, such as *A. kamchatica* and *A. l. petraea*, would be more cold tolerant than species like *A. thaliana* or *A. halleri* subspecies *gemmifera* (hereafter *A. h. gemmifera*), that have more southern distributions (Fig 2.1). The second goal of our study was to compare the cold tolerance of populations originating from different latitudes to determine if there was an inverse relationship between latitude and cold tolerance within each species. We tested populations from the four *Arabidopsis* species that were available in the University of

Alaska Fairbanks *Arabidopsis* collection for this comparison (Table 2.1). We predicted that northern populations would be more cold tolerant than southern populations.

MATERIALS AND METHODS

Arabidopsis species have a well-defined basal rosette, thus plants from high latitude populations are likely to have insulation from snow during winter (Fig 2.1). To examine variation in cold tolerance within the *Arabidopsis* genus, we assessed freeze damage before and after cold acclimation in plants from five taxa: *A. kamchatica, A. l. lyrata, A. l. petraea, A. thaliana,* and *A. h. gemmifera* (Table 2.1). A brief description of each taxon follows.

A. kamchatica (Fisch. Ex DC.) K. Shimizu & Kudoh is an allotetraploid, perennial that likely originated from the natural hybridization of *A. halleri* and *A. lyrata* (SHIMIZU-INATSUGI *et al.*, 2009). This species is found in eastern Russia, Asia, Alaska and Canada (HOFFMANN, 2005). Plants overwinter as a rosette and flower shortly after snowmelt. Although leaves are typically red and leathery after snowmelt, the leaves survive winter and may contribute resources needed for spring growth and flowering as there is no bulb or corm for resource storage. Plants are found primarily in disturbed areas, such as glacial till, roadsides, gravel pits and along creeks (personal observation) at elevations up to 3,500 m (AL-SHEHBAZ and O'KANE, 2002). The populations sampled for this study were mostly from North America (from British Columbia to Alaska), with two populations from Asia.

A. l. lyrata (L.) O'Kane & Al-Shehbaz is a diploid perennial that is found within temperate regions of the southeastern and central United States (HOFFMANN, 2005). Plants are found in woods, rocks, ledges, riverbanks, and sandy soils at elevations below 2,200 m (AL-SHEHBAZ and O'KANE, 2002).

A. l. petraea (L.) O'Kane & Al-Shehbaz is a diploid, perennial species. Populations of *A. l. petraea* can be found in some of the coldest regions occupied by any taxa in this genus, including northern and central Siberia (HOFFMANN, 2005). Its distribution

overlaps with *A. kamchatica* on its southeastern edge. Plants are found in rocky, sandy or gravelly ground as well as on tundra slopes and tussocks at elevations below 2,000 m (AL-SHEHBAZ and O'KANE, 2002).

A. thaliana is a diploid, annual species. *A. thaliana* has the widest climatic and geographic distribution of the taxa selected for our study (HOFFMANN, 2005). It was introduced to both coasts of the United States from Europe and is found in climates ranging from the Mediterranean to the Arctic Circle in northern Europe (HOFFMANN, 2005). *A. thaliana* has a continuous distribution from Ireland to western China but is also found in Taiwan and Japan, and ranges from New England to the southeastern United States (HOFFMANN, 2005). *A. thaliana* populations contain a mixture of summer-annual and winter-annual individuals (GRIFFITH *et al.*, 2004). Winter-annuals overwinter as a rosette and flower in spring. *A. thaliana* is found in disturbed, open and sandy habitats including meadows, rocky slopes, riverbanks and under shrubs at elevations below 4,250 m (AL-SHEHBAZ and O'KANE, 2002).

A. h. gemmifera is a diploid, perennial species found throughout Japan and Taiwan (HOFFMANN, 2005). *A. h. gemmifera* occupies regions that are warmer and more moist in summer than other *Arabidopsis* species (HOFFMANN, 2005). These plants are found in gravel or on grassy slopes, often in shaded or forested areas at elevations below 2,600 m (AL-SHEHBAZ and O'KANE, 2002).

Plant materials—

Arabidopsis kamchatica, A. l. lyrata, A. l. petraea, and A. h. gemmifera—

The sample sizes and populations that represent each taxon in this study (Table 2.1) were determined by the plant materials available from the *Arabidopsis* collection housed at the Institute of Arctic Biology Greenhouse, University of Alaska Fairbanks. Some were collected wild, either as full plants or as seeds; others were grown from greenhouse-gathered seeds. The plants were of different ages, but all had been grown in the greenhouse for at least 1 yr prior to testing.

Arabidopsis thaliana—

We obtained seeds from five accessions (Col-0, L*er*, Cvi, Seattle, British Columbia) of *A. thaliana* from the *Arabidopsis* Biological Resource Center at Ohio State University. Seeds from each inbred accession were considered to be genetically identical, so were statistically treated as if all were from the same individual.

Electrolyte leakage assay—

The measurement of electrolyte leakage in detached leaves is a common method to determine freeze tolerance in plants (HANNAH *et al.,* 2006). When cell membranes are damaged due to cold exposure, the cell contents leak out and are measured after being dispersed in water. Electrolyte leakage is expressed in terms of relative conductivity, where conductivity is measured after the initial exposure to cold temperatures, then the total conductivity is measured after the sample has been autoclaved to lyse the remaining cells (PRASIL and ZAMECNIK, 1998).

To minimize variation due to the hydration status of the leaves, we watered plants 24 hours before the leakage assays. For the electrolyte leakage assay, we removed one leaf from each plant for each temperature to be tested, and placed it in the bottom of a 10 x 13 mm glass-tube. We immersed the tubes in a NesLab circulating ethanol bath (Portsmouth, New Hampshire, USA), where they equilibrated at 0° C for 30 min and then we cooled the samples at a rate of 4° C/h. We paused cooling at -2° C and added small ice chips to each tube and ensured it was touching the leaf surface in order to nucleate the samples. We removed samples at 0, -2, -6, -10, -14 and -18° C. While temperatures during the growing season are unlikely to be this cold, this range of temperatures was selected because it included the estimated LT50 for non-acclimated plants based on previous studies (Armstrong *et al.*, in prep). The ice and leaves were allowed to thaw at 4° C overnight, after which we added 5.4 mL of distilled water to each tube. The tubes were shaken in a water bath at room temperature for 1 hour to homogenize the solution. We measured the initial electrolyte leakage (EL_I) using an Oakton CON6 conductivity meter (Vernon Hills, Illinois, USA), then the tubes were autoclaved and returned them to

the shaker to be shaken overnight at room temperature to release all of the contents of the cells. We measured the conductivity again for the 100% leakage measurement (EL_T). The relative leakage (EL_R) was calculated as (EL_I) /(EL_T).

After initial electrolyte leakage measurements, all plants were cold acclimated in a 4° C room with 8 h of soft-white fluorescent light per day for 4 weeks, then used for the acclimated electrolyte leakage trial. Methods for testing acclimated plants were identical except that we removed samples at 0, -4, -11, -18, -25, -32 and -35° C. We chose this range because our preliminary studies showed that cold acclimated plants suffered very little damage at the temperatures used for non-acclimated plants. We performed three replicate measurements for each plant.

Data analysis—

To estimate the point at which 50% of electrolyte leakage would occur (LT50), we fit the data to a sigmoidal curve in which the relative leakage EL_R at the temperature T follows a two-parameter logistic model:

$$EL_R = 1 - 1 / \{1 + \exp[-(T - d) / s\},$$

where the inflection point, d, gives the LT50 estimate, and s is a scale parameter. With this method, the lower asymptote of the curve approaches 0 at temperatures that are too warm to cause damage to the leaf tissue, the upper asymptote approaches 1 at temperatures at which the most cells would be lysed. The sigmoidal curve fitting method has been widely used in cold tolerance reporting with various methods used to fit the data to the curve including the logistic function, Gompertz function (HAO *et al.*, 2009) and functions built into GraphPad Prism3 (HANNAH *et al.*, 2006; LE *et al.*, 2008). A benefit of the Gompertz function is that it allows both asymptotes to be approached by the curve asymmetrically unlike the logistic function where the asymptotes are approached symmetrically. However, we were not able to achieve statistical convergence with the Gompertz function with our data set, therefore it was necessary to use the logistic function.

Some methods of reporting freeze damage such as simply calculating the LT50 to be 50% of the maximum leakage obtained from measurements (LIPP *et al.,* 1994; LOIK and REDAR, 2003) don't take into account all of the data because they are based on a single point rather than including the electrolyte leakage data from multiple temperatures to estimate the LT50. Estimates based on a single point are less accurate than the method we used. Other studies report only the percentage of electrolyte leakage (HASDAI *et al.,* 2006; NUNES and SMITH, 2003) or use the percentage leakage as a direct way to estimate survival (NAGAO *et al.,* 2008). With these methods, it wouldn't be possible to make direct comparisons across multiple species or populations. Although the large confidence intervals produced by our sigmoidal curve fitting method to estimate LT50 would seem to be a flaw of the method (Fig 2.2), it is more statistically accurate than more simplistic methods that estimate LT50 with no confidence intervals.

Population structure within taxa and the sigmoidal nature of the data made it necessary for us to use a non-linear mixed-effects model to analyze the electrolyte leakage data. We used *nlme* package (PINHEIRO and BATES, 2000) implemented in R Statistical Environment (R DEVELOPMENT TEAM, 2011). The dependent variable was the relative electrolyte leakage. For among taxa comparisons, our independent variables, taxon and acclimation treatment, were treated as fixed effects; and population, family, individual, and date of EL measurement were modeled as random effects. In this model, these independent variables were allowed to have linear effects on the two parameters of the logistic function. We first conducted a model selection (FARAWAY, 2006) to include the relevant random effects by Akaike Information Criterion (AIC), following Pinheiro and Bates (2000). In order to test the significance of fixed effects, i.e., whether acclimation and/or taxa influenced the shape of the electrolyte leakage response curve, we performed likelihood ratio tests to compare models with and without a fixed effect (including the interaction of taxon and acclimation effects). The test statistic reported for the likelihood ratio tests is D, where D is twice the difference in the log-likelihoods of

two models: D = -2 ln (likelihood for a simpler model / likelihood for a model with more parameters).

Then, in order to estimate the confidence intervals of the estimated parameters for each taxon, we conducted non-parametric bootstrapping for 1,000 iterations (EFRON and TIBSHIRANI, 1998; FARAWAY, 2002). In the bootstrap, each observation (the relative electrolyte leakage measurement) was the unit of resampling, and the total number of observations was constrained to match the data. Briefly, non-linear mixed-effects models containing the relevant independent variables as described above were fitted to each bootstrapped data set, and confidence intervals were calculated from the distribution of estimated parameters. We checked for normality and homogeneity of errors by inspections of plots of residuals against fitted values and Quantile-Quantile (QQ) plots. If the assumptions were violated, we attempted to fit an extended non-linear mixed-effect model, where appropriate variance functions can be used to model heteroscedasticity of within group errors (Chapter 8, (PINHEIRO and BATES, 2000). The variance function we used was 'varPower(fixed=0.5, form= ~ fitted(.) - fitted(.)^2))'. However, if this correction failed to converge due to added the complexity, we were forced to use the original models without the correction.

For within taxon comparisons, our methodology for estimating the LT50 for each population was identical except that the independent variables were family, individual, and date of electrolyte leakage measurement as the random effects and population as the fixed effect. For *A. h. gemmifera,* only one population was available for testing, therefore this taxon was excluded from the population-level comparisons.

We used analysis of covariance to test whether acclimation treatment and the latitude of origin of the population influenced LT50, as estimated for each population with the non-linear mixed-effects models described above. The independent variables were latitude, taxon, and acclimation. We chose a model by AIC-based stepwise selection (HASTIE and PREGIBON, 1992; VENABLES and RIPLEY, 2002), implemented in *step()* function of R (R DEVELOPMENT TEAM, 2011). Box-cox transformation of the dependent

variable was used to reduce non-normal distribution of residuals (BOX and COX, 1964; FOX and WEISBERG, 2011). All statistical analysis was done in the R Statistical Environment (R DEVELOPMENT TEAM, 2011).

RESULTS

Differences among taxa—

Before acclimation, the cold tolerance was relatively similar among populations and taxa, within 3.5° C of each other, but acclimated LT50 values were much more variable (Fig 2.2). However, within both pre- and post- acclimation, the differences in LT50 among taxa were statistically significant (pre: D=10.138, df=11, p=0.0382; post: D=9.534, df=12, p=0.049). Because it is not feasible to do a post hoc comparison between species, we chose to compare the LT50 values of different species using bootstrapped confidence intervals. In order to assess whether taxa differed in LT50, we analyzed pre-acclimation and post-acclimation data separately. The capacity for cold acclimation varied among the five taxa of *Arabidopsis* tested. *A. kamchatica, A. l. lyrata* and *A. l. petraea* all had a high capacity for increased cold acclimation (-10.0°, -6.7° and -8.0° C, respectively), while *A. thaliana* and *A. h. gemmifera* showed a low capacity cold acclimation (-2.8° and -1.7° C, respectively). *A. kamchatica* had the lowest LT50 of the taxa tested after acclimation, suggesting that it may be able to withstand the coldest winter temperatures (Fig 2.2).

Variation within each taxa—

The likelihood ratio test detected significant interaction of taxa and acclimation effects on cold tolerance, as measured by LT50 (D=11.629, df=26, $p < 0.02$). Therefore, we tested the effect of acclimation on LT50 by analyzing each taxon separately. All taxa except for *A. h. gemmifera* exhibited a decrease in LT50 following cold acclimation (Fig 2.2 and 2.3: *A. kamchatica*: D=77.513, df=10, p=<0.0001; *A. l. lyrata*: D=29.658, df=5,

p=<0.0001; *A. l. petraea*: *D*=145.867, df=7, p=<0.0001; *A. h. gemmifera* (*D*=1.10, df=5, p=0.2942; *A. thaliana*: *D*=16.965, df=5, p=<0.0001).

In *A. kamchatica,* all populations except for the one at 63° N (Rainbow Ridge) had a high acclimation capacity, measured as the difference between non-acclimated and acclimated LT50 values (Fig 2.3A and B). The LT50 estimates for several populations of *A. kamchatica* (Rainbow Ridge, Exit Glacier and Thompson Pass) had wide confidence intervals (Fig 2.3B). This is partially because several LT50 estimates were based on single plant assays with three replicates per plant for these populations. Three replicate leaves were tested for each individual because this takes variation among leaves into account when estimating the LT50 for each individual. For each replicate, care was taken to select leaves of the same size and life stage, however slight differences between individual leaves on a single plant would lead to differences in electrolyte leakage, and thus larger confidence intervals if the entire population is based on three measurements from a single individual.

A. kamchatica had the widest latitudinal gradient of all the taxa tested , ranging from 24° to 65° N, with two of the populations originating from outside North America (Table 2.1). However, the Japanese population was excluded from further analysis because of problems with LT50 estimation: non-acclimated and acclimated LT50 for this population were estimated to be -105.1° C and -28.8° C respectively, both of which are below the lowest temperature tested (data not shown). Due to the nature of non-linear mixed-effect models, estimation of an inflection point (LT50) that is outside of the measured range is highly unreliable.

Variation within *A. thaliana* was very low compared to the other taxa. Before cold acclimation all of the LT50 values for *A. thaliana* were within 1° C of each other. While there was more variation after acclimation, all LT50 values were still within 4° C of each other (Fig 2.3H).

A. l. petraea from the far north had some of the lowest acclimated LT50 values (Fig 2.3F). Yet surprisingly, plants from two different northern populations, which are

geographically very close (both in Iceland at ~65° N), showed very different acclimated LT50 values. Based on the single plant available for testing, it appears that only the Reykjavik population had a high capacity for cold acclimation, the other populations of *A. l. petraea* remained almost the same before and after acclimation. The non-linear mixed-effect model did not converge with the non-acclimated data for the population from Braemer, Scotland so it was excluded from the non-acclimated analysis.

To examine overall latitudinal variation in cold tolerance, we measured freeze damage in several populations from four out of five of the *Arabidopsis* taxa before and after cold acclimation, and used ANCOVA to investigate the relationship between latitude and estimates of LT50. AIC-based stepwise selection chose a model with taxon and acclimation effects ($F_{4, 42}$=2.92, $p < 0.03$ for taxon effect and $F_{1, 42}$=15.59, $p < 0.001$ for acclimation effect), indicating that there were differences among taxa, and that acclimation influenced LT50. However latitude did not have a statistically significant effect on LT50 among populations. The relationship between LT50 and latitude was also not statistically different between the acclimation treatments. There was, however, more extreme variation in the capacity for cold acclimation among northern populations than southern populations (Fig 2.4).

DISCUSSION

Differences among taxa—

We found that cold tolerance increased after acclimation for all but one taxon and there was variability among taxa in their capacity for cold acclimation. Taxa with northern distributions, *A. kamchatica* and *A. l. petraea,* generally had higher acclimation capacity and overall cold tolerance than two of the southern taxa (*A. thaliana* and *A. h. gemmifera*). However, *A. l. lyrata*, has a more southern distribution and was very cold tolerant. This North American subspecies is thought to be recently derived from the very cold tolerant subspecies *A. l. petraea* (WRIGHT *et al.,* 2003) via a founder event from

Europe. Thus a shared genetic background with *A. l. petraea* and its past distribution, rather than this taxon's current geographical distribution, may explain the high cold tolerance of *A. l. lyrata*. Alternatively, *A. l. lyrata* may be exposed to as much cold as the more northern taxa. In the coldest climates, such as interior Alaska, *A. kamchatica* is insulated by snow throughout the winter, in 2008 recorded temperatures under the snow didn't drop below -5.5° C, even when the air temperatures plummeted below -40° C (data not shown). However, in some regions where *A. l. lyrata* grows, such as on the shores of Lake Michigan, the snow frequently melts, and the plants would be directly exposed to the air in a cold snap.

Our prediction that taxa with northern distributions would be more cold tolerant than those with southern distributions was mostly supported by our results, but additional factors, such as evolutionary history, might also influence acclimation capacity. While our study design did not measure the fitness characteristics that would allow us to conclusively determine that there is a physiological cost to maintaining cold tolerance, the retention of this trait in populations from regions that do not experience extreme cold indicates that the cost of maintaining the ability to tolerate cold temperatures might not be high enough to incur strong selection against this trait. It would be necessary to determine the actual temperatures leaves are experiencing to correlate any fitness differences with cold exposure in order to be able to measure a cost of cold tolerance. This is likely why few studies have addressed this question thus far (JACKSON *et al.*, 2004); however this type of study would be valuable for understanding more about range boundaries and the differences among taxa.

There was overlap in the range distributions of many of the taxa we tested (Fig 2.1). When we compared samples of different taxa from similar latitudes (Fig 2.4), some were more cold tolerant than others. For example, we included populations from where the ranges of *A. thaliana* and *A. kamchatica* overlap in British Columbia and found that the LT50 of *A. kamchatica* was 2.2° C colder before, and 13.3° C colder after acclimation than *A. thaliana* (Fig 2.2). *A. thaliana* also overlaps with the range of *A. l. lyrata*

(HOFFMANN, 2005); when we compared populations from within 3° latitude, the LT50 of *A. l. lyrata* was 2.7° C colder before and 7.5° colder after acclimation than *A. thaliana*. These results appear to be concordant with the hypothesis that the overall taxon distribution and the evolutionary history of a population may be more important than the current location of a particular population.

Differences within taxa—

Unexpectedly, we did not find that populations from high latitudes had a greater capacity to cold acclimate. The reason that we did not find a linear latitudinal trend could be that we need more population samples per taxa than what was available. Or alternatively, as we mentioned above, cost of cold tolerance could be small, therefore, there may not have been enough time for adjustments in cold tolerance among populations within a taxon. Plant species with broad distributions often show intra-specific variation in cold tolerance (DAVEY *et al.*, 2009). It is difficult to predict how plants will respond to changes in climate but natural variation for traits such as cold tolerance may be important for plant adaptation to rapidly changing climates (DAVEY *et al.*, 2009; SAKAI and LARCHER, 1987). In many parts of the world, the length of the growing season and annual temperature régimes have changed with global climate change (SIERRA-ALMEIDA *et al.*, 2009).

Another possible explanation of failure to find a linear latitudinal trend is that the relationship could be obscured by factors other than latitudes such as winter precipitation and the pattern of snow melt-freeze cycle. Snow cover and cold acclimation provide protection for many taxa during winter, therefore, survival of plants in low temperatures during the growing season may be more difficult for plants than over-winter survival (SIERRA-ALMEIDA *et al.*, 2009). For example, in the northernmost population of *A. l. lyrata* in our study, there was little capacity for cold acclimation indicating that the Wisconsin population may need to have some level of cold tolerance year-round. On the other hand, the North Carolina population of *A. l. lyrata* had a high capacity for cold

acclimation, which may indicate that during most of the year cold tolerance is not important; but for over-winter survival in the mountains, cold tolerance is necessary.

We suspect that unusually low amount of freeze damage (less than 60% for all temperatures tested) may have been an artifact of measurement procedure. The leaves on plants from this population were densely covered with trichomes which could have prevented the ice chips from touching the leaves and nucleating the leaves, this could have allowed leaves to supercool and potentially influenced the amount of freeze damage. More populations with multiple individuals would need to be tested to better determine the differences between the Asiatic and the North American populations.

A. thaliana has the widest regional distribution of the taxa included in this study and thus the conditions that individual populations must tolerate were quite variable. For this reason we expected to find a cline in cold tolerance, however there was not. This may be due to the origin and limited number of accessions included in our study, or slight differences in methodology because other studies have documented a latitudinal cline in cold tolerance both before and after acclimation (HANNAH *et al.,* 2006; ZHEN and UNGERER, 2008) in *A. thaliana*. Our methodology was similar to other studies, except that our plants were cold acclimated for four weeks, while their plants were only acclimated for two weeks (HANNAH *et al.,* 2006). Nonetheless, it is reassuring that even with differences in methodology, our estimates are similar to the previous study; thus validating our methods (HANNAH *et al.,* 2006). Three accessions were common between our studies, and the acclimated LT50 values for Col-0 were very similar, and for Cvi and L*er*, our values are only approximately 2° C colder than values reported by Hannah *et al.* (2006).

We found variation among and within species in our survey of *Arabidopsis* taxa, but a more thorough survey will be required to examine latitudinal gradients. Low variability in cold tolerance before acclimation and increased variability after acclimation were also found among *A. thaliana* accessions (HANNAH *et al.,* 2006) and in *Petunia* species (WALWORTH and WARNER, 2009), indicating that differences in acclimation capacity at

both the population and species level may be an interesting topic for future climate change and range boundary research. Few studies have previously examined cold tolerance within and among taxa. We were fortunate to have such an extensive collection of *Arabidopsis* plants from around the world. Although we had 12 populations of *A. kamchatica*, collections from other taxa were more limited and often did not include populations from the entire taxon range.

Summary—

Our results suggest that latitudinal clines in cold tolerance are not universal, but that within and among closely related taxa there is variability in cold acclimation capacity. Research on among taxa differences will help us better understand natural variation; this is useful for a variety of agricultural and plant management applications. In the face of global climate change, empirical data on natural variation, especially in cold tolerance, may be useful for predicting future range shifts in the far north and understanding the threats of potential invasion from exotic species. High latitude species may face competition and possibly invasion at their southern range boundaries if warming temperatures make northern latitudes more favorable for new colonizers (CALLAGHAN *et al.*, 2004).

Much is known about cold tolerance in plants, largely because of the extensive amount of research on *A. thaliana* (HANNAH *et al.*, 2006; THOMASHOW *et al.*, 2001; ZHEN and UNGERER, 2008), but relatively few studies have examined intra-specific cold tolerance along latitudinal gradients (ZHEN and UNGERER, 2008). Even fewer studies also examined cold tolerance in multiple populations from several different taxa from within the same genus. We compared cold tolerance among *Arabidopsis* taxa and looked at latitudinal variation within each taxon to assess how variable this essential trait is among closely related taxa. This is a first look at cold tolerance in other members of the *Arabidopsis* genus, and several taxa have much greater cold tolerance and a greater capacity for cold acclimation than *A. thaliana*. Since molecular tools are easy to transfer

from *A. thaliana* to its relatives, these taxa are likely to be excellent candidates for studying both the molecular and ecological aspects of cold tolerance.

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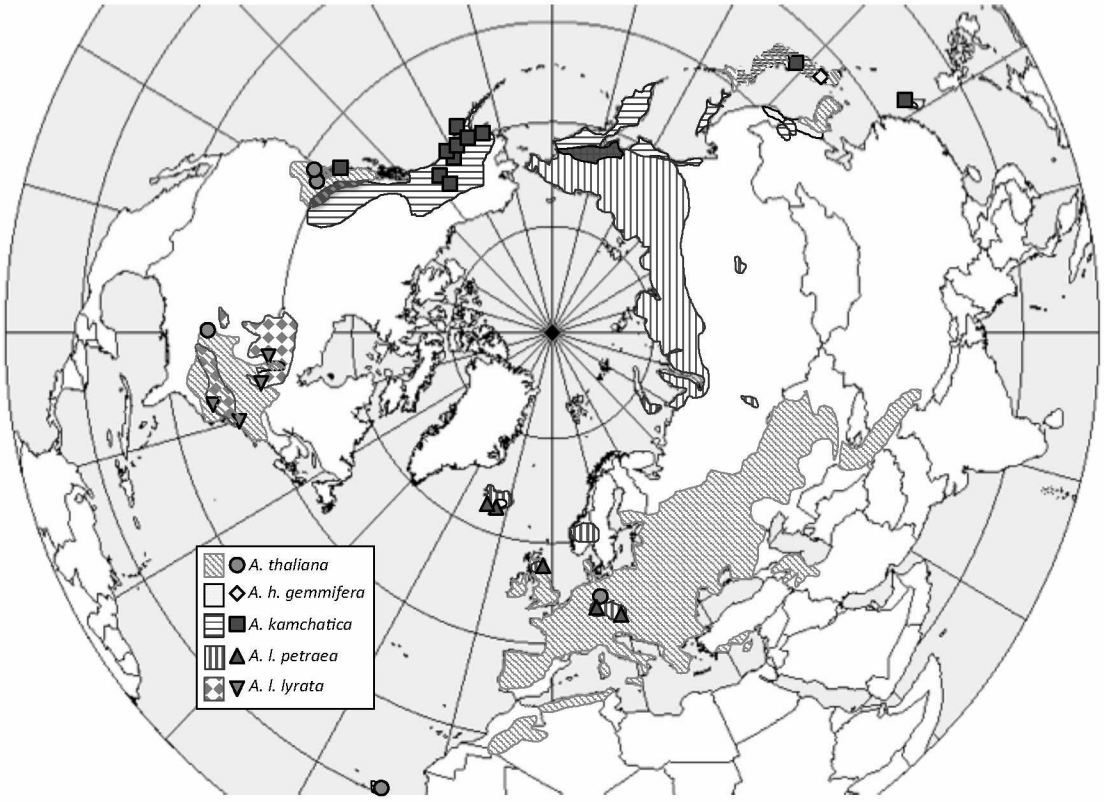
Table 2.1 *Arabidopsis* species, locations of population tested for cold tolerance, and number of individuals per population.

Species	Source locality	Latitude	Longitude	Number of individuals
A. kamchatica	Taiwan	24° N	121° E	2
A. kamchatica	Lake Biwa, Japan	35° N	136° E	1
A. kamchatica	Strathcona Park, BC, CAN	49° N	125° W	12
A. kamchatica	Kodiak, AK, USA	57° N	154° W	1
A. kamchatica	Portage Glacier, AK, USA	59° N	161° W	12
A. kamchatica	Thompson Pass, AK, USA	60° N	149° W	1
A. kamchatica	Exit Glacier, AK, USA	60° N	148° W	3
A. kamchatica	Goodnews Bay, AK, USA	61 °N	160° W	1
A. kamchatica	Rainbow Ridge, AK, USA	63° N	145° W	1
A. kamchatica	Pratmigan Creek, AK, USA	65° N	145° W	12
A. lyrata ssp. *lyrata*	Cedar Mountain, NC, USA	35° N	82° W	1
A. lyrata ssp. *lyrata*	New York, USA	41° N	74° W	1
A. lyrata ssp. *lyrata*	Presque Isle, PA, USA	42° N	80° W	1
A. lyrata ssp. *lyrata*	Bailey's Harbor, WI, USA	44° N	87° W	1
A. lyrata ssp. *petraea*	Plech, Germany	49° N	11° E	1
A. lyrata ssp. *petraea*	Exeter McNair, England, UK	50° N	3° E	1
A. lyrata ssp. *petraea*	Braemer, Scotland, UK	57° N	3° W	1
A. lyrata ssp. *petraea*	Esja Mountain, Iceland	64° N	21° W	1
A. lyrata ssp. *petraea*	Reyjkavic, Iceland	64° N	21° W	1
A. thaliana	Cape Verde Islands	15° N	23° W	1
A. thaliana	Col-0 Columbia, MO, USA	38° N	92° W	1
A. thaliana	Seattle, WA, USA	47° N	122° W	1
A. thaliana	British Columbia, CAN	49° N	123° W	1
A. thaliana	Landsberg, Germany	52° N	10° E	1
A. halleri ssp. *gemmifera*	Fujita (Gifu), Japan	34° N	136° E	3



http://tao-dnd.blogspot.com/2011/09/projection.html

Figure 2.1 Map of range distributions for *A. kamchatica, A. l. lyrata, A. l. petraea, A. thaliana,* and *A. h. gemmifera*. Map modified from Hoffmann, 2005.

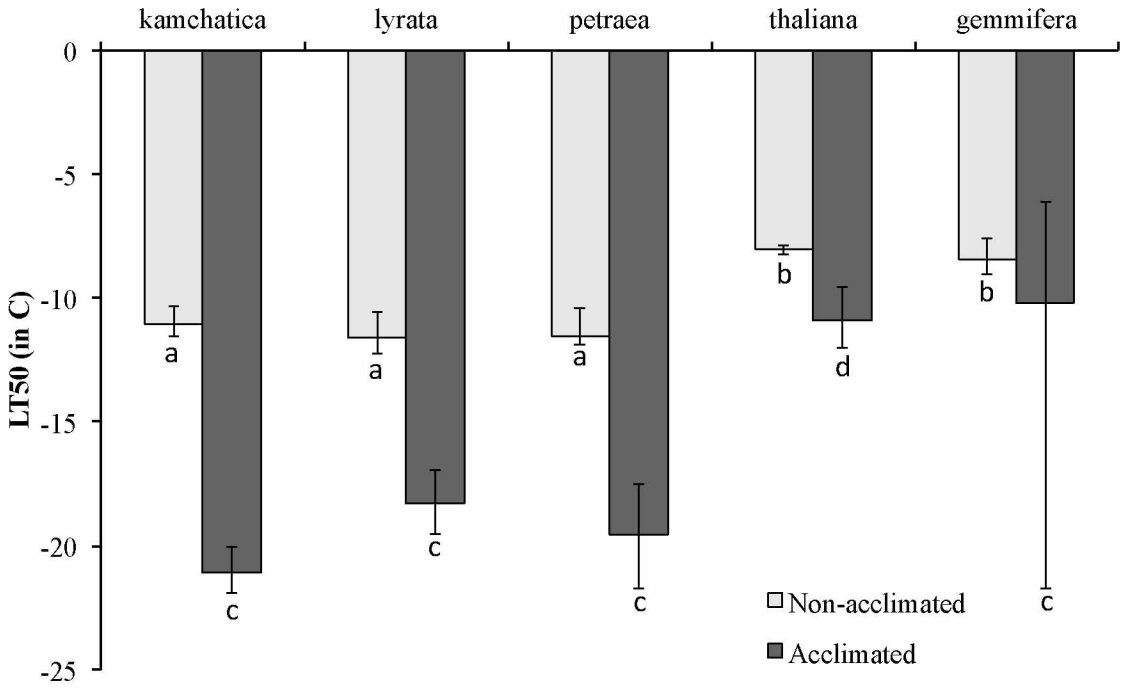


Figure 2.2 The LT50 of 5 *Arabidopsis* species before (grey bars) and after (black bars) cold acclimation. LT50 values were estimated using a logistic curve fitted by non-linear mixed modeling analysis on electrolyte leakage measurements. Means with the same letters were not significantly different. Error bars represent 95% confidence intervals around the means.

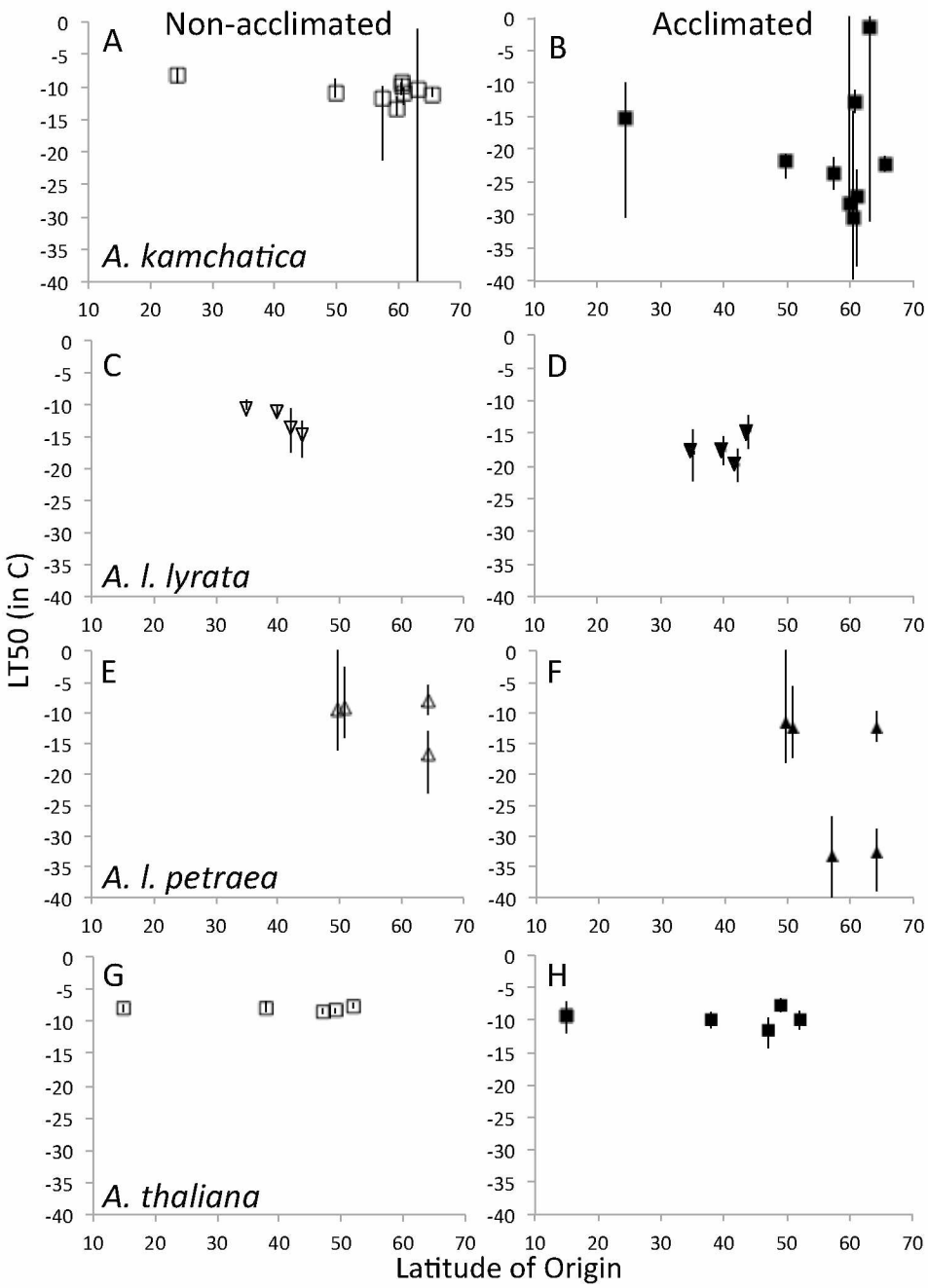


Figure 2.3 LT50 value of each population of *A. kamchatica* (A, B), *A.l. lyrata* (C, D), *A.l. petraea* (E, F), *A. thaliana* (G, H), and the latitude of population origin before (open symbols) and after (closed symbols) cold acclimation. Error bars represent 95% confidence intervals around the means.

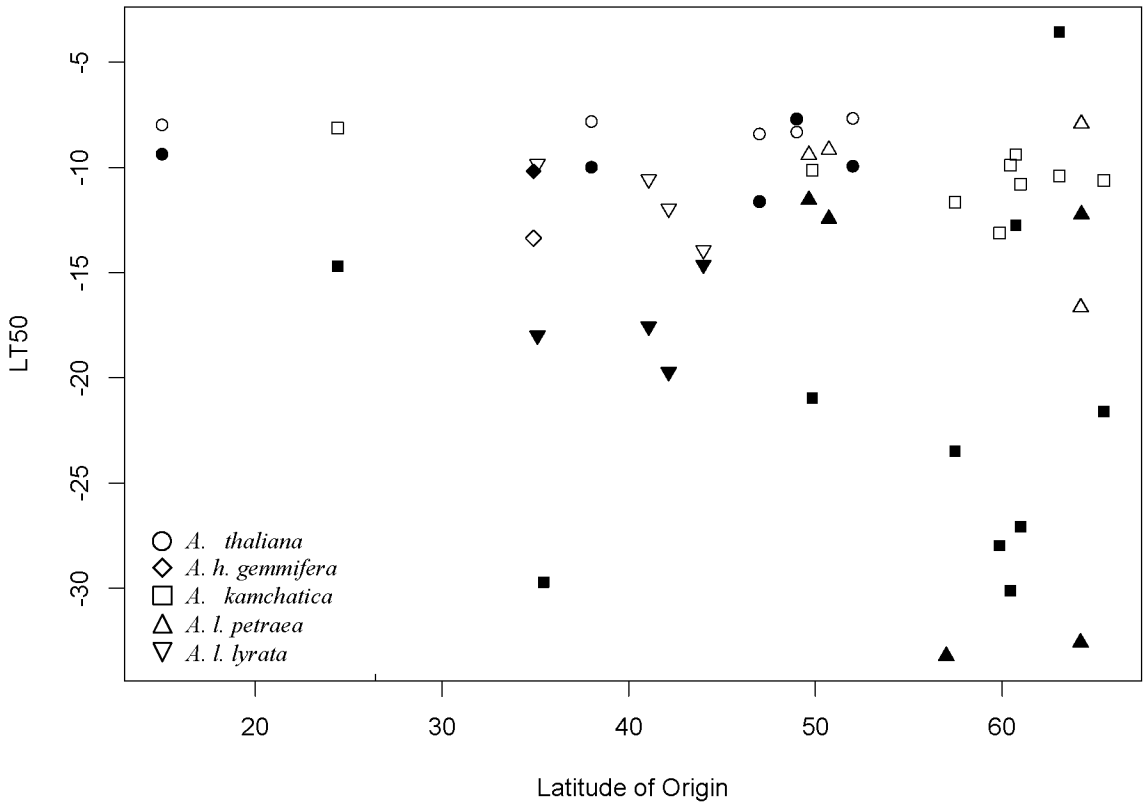


Figure 2.4 LT50 of populations from *Arabidopsis* species before (open symbols) and after acclimation (closed symbols) by latitude of population origin.

GENERAL CONCLUSIONS

Our study of cold tolerance in *Arabidopsis* demonstrated that cold tolerance and acclimation capacity varies among closely related species. We predicted that there would be a latitudinal cline within species based on a previous study in *A. thaliana* (ZHEN and UNGERER, 2008), which found a strong relationship between latitude of origin and cold tolerance. However the origin of the population was not a significant factor in cold tolerance capacity for any of the species in our study. Other studies have demonstrated that latitudinal clines in cold tolerance are not universal, likely because the actual temperatures plants experience are not totally correlated with latitude because of differences in microhabitat. For example, in a study of *Acer platanoides* (Norway maple) it was found that during fall there was some variation in cold hardiness amongst populations, however by early winter there were not differences among the populations (PAGTER *et al.*, 2010). In contrast, for our *Arabidopsis* study there was variation amongst populations in cold tolerance both before and after cold acclimation, just no strong latitudinal trends. It may be that factors such as frequency of freeze-thaw cycles or snow depth have a stronger influence on cold tolerance than latitude of origin because with the insulation provided by snow cover plants are unlikely to experience extremely cold temperatures, unfortunately our study design did not allow us to address this question.

We found that *A. kamchatica* and *A. thaliana* both have the capacity to avoid summer frosts by supercooling. However, after cold acclimation, the freeze damage (LT50) in *A. kamchatica* is much lower than the supercooling point, which indicates that this species is able to tolerate freezing in the absence of external nucleation. In *A. thaliana* freeze damage did not exceed the supercooling point, indicating that the over-winter strategy for this species is to avoid freezing by supercooling. This result agrees with another study that examined both freeze damage and supercooling in *A. thaliana* (REYES-DIAZ *et al.*, 2006). Freeze avoidance via supercooling is thought to mainly be an effective strategy in climates where a few degrees of freeze protection would be sufficient (WEIGEND and LUEBERT, 2009) and for the extreme temperatures of northern climates this would not be adequate protection. However this strategy has also been

found in Antarctic peralwort, *Colobanthus quitensis* where the supercooling point was lower than the LT50 before and after acclimation (BRAVO *et al.,* 2001). Both *A. thaliana* and *C. quitensis* appear to have little capacity for cold acclimation.

We found that acclimation increased cold tolerance in all but one species tested, and the recently diverged species *A. kamchatica*, *A. lyrata* subspecies *lyrata*, and *A. lyrata* subspecies *petraea* were most cold tolerant while also having the highest acclimation capacity. Extensive research has been done with *A. thaliana* on the physiological and genetic underpinnings of cold tolerance in plants (GILMOUR *et al.,* 1988; NAGAO *et al.,* 2008; THOMASHOW, 1999; ZHEN and UNGERER, 2008) while ours is the first study to include other *Arabidopsis* species. We found that several species have much greater cold tolerance and a greater capacity for cold acclimation than *A. thaliana*. Since molecular tools are easy to transfer from *A. thaliana* to its relatives, these taxa are likely to be excellent candidates for studying both the molecular and ecological aspects of cold tolerance.

Cold tolerance has been studied for well over 100 years, largely with the goal of understanding and improving crop species in order to increase suitable locations for agriculture (THOMASHOW, 1999). While some advances have been made in the identification of cold hardy species for use in breeding programs (CHAT, 1995), only modest improvements have been made in the development of crop freezing tolerance (THOMASHOW, 1998), and freeze damage remains a significant source of economic loss to the agriculture industry. Developing more tolerant crops may require genetic bioengineering of transgenic plants rather than traditional breeding methods (JAN *et al.,* 2009). Understanding interspecific differences in cold tolerance may lead to a better understanding of natural variation within species; this is useful for a variety of agricultural and plant management applications. It has been predicted that with global climate change there will also be an increase in the frequency of extreme weather events and changing temperature and moisture regimes which may further increase agricultural losses (BERTRAND and CASTONGUAY, 2003; TRNKA *et al.,* 2011). Learning how plants in the far north survive frost events during the growing season may help us to better

engineer crops to survive extreme weather events, either through breeding programs or bioengineering of transgenic crops.

The length of the growing season and annual temperature régimes have already changed in some parts of the world, and unseasonable events like frosts during the growing season may become more common in some regions with continued climate changes (SIERRA-ALMEIDA *et al.*, 2009). In the face of global climate change empirical data on natural variation, especially in cold tolerance, may be useful for predicting future range shifts in the far north and understanding the threats of potential invasion from exotic species. High latitude species may face possible invasion and subsequent competition at their southern range boundaries if warming temperatures make northern latitudes more favorable for new colonizers (CALLAGHAN *et al.*, 2004). Plants must adapt to new conditions in order to expand their range and tolerate a changing climate. The necessity for high latitude plants to tolerate a wider range of temperatures means that fewer species are able to adapt to northern climates (CRIDDLE *et al.*, 1994). Studying the evolution of cold tolerance among related species across a wide latitudinal range helps us to understand both historic and future species range expansions and contractions brought about by changes in climate.

While our study design did not specifically address the fitness characteristics that would allow us to conclusively determine that there is a physiological cost to maintaining cold tolerance, the retention of this trait in populations from regions that do not experience extreme cold indicates that the cost of maintaining the ability to tolerate cold temperatures might not be high enough to incur strong selection against this trait. Few studies have addressed this question thus far (JACKSON *et al.*, 2004); this type of study would be valuable for understanding more about range boundaries and the differences among species.

Our study found differences in cold tolerance among *Arabidopsis* species. A further investigation of the over-winter strategy among additional members of this genus would be informative, particularly if this included measurements of the temperatures that plants actually experience in nature because this would allow us to determine how

conserved the freeze tolerance trait is in closely related species where the range distributions are well understood. It would also be interesting to determine if *A. kamchatica, A. l. lyrata* and *A. l. petraea* share the same over-winter strategy. If so, this may help explain why these species have far more cold tolerance than *A. thaliana* and *A. h. gemmifera*. Given that latitudinal clines have been found in several other species, it would also be beneficial to do a more thorough investigation of cold tolerance within each species to see if including additional populations from varying locations shows a different result than our study.

Our project's larger goal was to develop a better understanding of cold tolerance in the *Arabidopsis* genus. We integrated methods for measuring supercooling and freeze damage that could be easily implemented to examine over-winter strategy in additional *Arabidopsis* species or other herbaceous plants. The practice of studying cold tolerance while integrating the evolutionary relationships between species is a relatively new (BYARD *et al.*, 2010) and exciting direction for future studies.

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